Bovine epithelial cell responses to colonisation by *Escherichia coli* O157:H7

By

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Declaration

I declare that this thesis and the experiments described therein are my own work, except where otherwise indicated. No part of this thesis has been submitted for a degree at this or any other University. All sources of information have been acknowledged by means of reference.

Neil Paton August 2008

Abstract

E. coli O157:H7 was the first identified pathogen of a group that have come to be referred to as Enterohaemorrhagic *E. coli* (EHEC) and was identified in 1983 as being associated with Haemolytic ureamic syndrome (HUS). This bacterium carries the potent cytotoxin Shigalike toxin (Stx), also known as verotoxin, which through the inhibition of protein synthesis causes cell necrosis in the endothelium of the renal vasculature and this leads to the triad of symptoms – renal failure, thrombocytopenia and microangiopathic haemolytic anaemia. EHEC is also associated with Haemorrhagic colitis in humans as endothelium of both the colonic vasculature and the renal system express Gb3 receptors. These receptors bind the toxin and internalisation of the toxin allows the inhibition of protein synthesis.

EHEC is a food borne zoonosis and its reservoir host is the bovine and faecal contamination of the environment, the food chain and direct contact with cattle are the most recognised routes for human infection to occur. The bovine host is asymptomatically colonised in the field and detection and removal is problematic, any future intervention strategy to remove this pathogen from the national herd is likely to be expensive and labour intensive.

EHEC has a number of virulence factors that are involved in colonisation and proinflammatory responses. These were examined in a bovine model system. The flagellum was the only virulence factor that produced a proinflammatory response when measured by quantitative and traditional RTPCR. Commensal bacteria were unable to produce a response although one motile strain was included in the panel and was presumed to express flagella which were shown to be pro inflammatory when associated with EHEC.

In the host animal there is limited evidence for a pathological effect and to elucidate the mechanisms that explain this lack of response to pathogenic effects microarray was utilised to tease out these mechanisms. The data produced identified a limited set of genes that were differentially regulated including CyclinC Angiopoetin-1 like protein, Jumonji domain containing protein 2B, Zinc finger protein 161 and Est-1plike protein, all of which were thought to be involved in cell cycle regulation. Quantitative RTPCR was unable to confirm the data from the array; further work is therefore required to determine whether colonisation does in fact alter the expression of these genes.

EHEC was therefore hypothesized to alter the proliferation rate within the epithelium and an immunohistochemical approach was used to assess this. Proliferating cell nuclear antigen (PCNA) was used as a marker to identify replicating cells and counts of cells in the epithelium demonstrated a reduction in proliferating cells in colonised epithelium. Further analysis suggested that retinoblastoma protein was a central protein that was involved in pathways influenced by the proteins already outlined. IHC was used to study this protein and differences in the number of cells expressing this protein and localisation within the cells. Retinoblastoma appears to be retained in the cytoplasm in colonised cattle which limits its ability to induce proliferation through release of E2F.

It is suggested that *E.coli* O157:H7 can manipulate the epithelial cell proliferation rate in the bovine host and increases the time that the bacterium is retained in the host. It was hoped that microarray data and QtRT-PCR would identify proteins involved in this phenotype but the lack of support from the real time data for targets identified by the array makes it impossible to conclude that these proteins are defiantly involved. This increase in time allows for a greater chance of spread to other host animals within the herd. Further

work to clarify the details of this pathway will allow interventions which limit colonisation in the National herd to be designed.

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Common abbreviations

A/E	Attaching and effacing
Gb3	Globotriaosylceramide
CFU	Colony forming units
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic E. *coli.*
Esp	*E. coli* secreted proteins
HACCP	Hazard and critical control point
HC	Haemorrhagic Colitis
HUS	Haemolytic Ureamic Syndrome
LCM/LCMD	laser capture microdissection
LEE	Locus of Enterocyte effacement
LT	Labile toxin
Nal	Nalidixic acid
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
ST	Stable toxin
STEC	Shiga-toxigenic *E.coli*
VTEC	Verotoxigenic *E. coli*

Chapter 1

Introduction

The Calves.

1. Introduction

Food borne infections are a concern for the farming industries as these diseases cause economic disruption and human health problems in significant numbers. There are a variety of pathogens involved in these diseases which may be viral, fungal, parasitic or bacterial in origin. These pathogens can contaminate the human food chain at a variety of points from the farm through to the final preparation and presentation of food. Food hygiene standards are of critical importance in this as breakdown of these standards may allow introduction of pathogens into the food chain. Although gastrointestinal infections are frequently associated with ingesting the organism through meals other infection routes occur including direct contact and fomite transmission. Pathogens that can cause gastrointestinal disease include the viruses rotavirus and Norovirus, the protozoa *Giardia* and *Cryptosporidium,* occasionally the fungal pathogen *Candida albicans* can cause opportunistic infection (Margolis et al., 1990). Bacteria which can cause enteric disease include *Campylobacter, Salmonella* and others including *E. coli* types and these bacteria are frequently involved in food associated disease.

Food borne pathogens have a variety of life cycles and inhabit a variety of hosts and these pathogens can also cause a variety of pathologies but clinical signs are virtually indistinguishable. Disease is primarily signified by the presence of diarrhoea, the presence of vomiting is not unusual with other clinical signs being noted depending on the pathogen involved. This means that diagnosis of a specific disease associated with a pathogen requires laboratory techniques to identify the specific pathogen involved.

The management of patients with vomiting and diarrhea caused by food borne disease relies on the replacement of fluid and electrolyte losses from the intestine. The common presentation in all these diseases is a derangement of fluid balance and ionic losses

within the gut (Fontaine, 1996) and the specific mechanism by which this occurs varies between pathogens.

Identification of the pathogen is useful in managing the more severe diseases but makes little change in the management of to the majority of simple cases. However it can be very important in the tracing and elimination of the source as knowledge of the pathogens lifecycle may allow the identification of likely places where it may have infected patients.

There are several bacterial pathogens that can infect human pathogens from the food chain of which the most well known are *Salmonellae*, *Campylobacter* and *E. coli*. These bacteria are found in wildlife, commonly water birds, although other farmed species may be colonised and compromise the food chain, with colonised animals which may not be symptomatic being important sources of contamination.

1.1 Enteric diseases of humans.

There are a number of pathogens that can cause diarrhea in humans and these can frequently be acquired from animals, such diseases are termed zoonoses and can cause disease among animals as well as humans or may be carried apparently as part of the resident microbial flora. Enteric diseases can be viral, bacterial or protozoal and may be acquired through both direct and indirect contact with animal faeces. Examples of these pathogens include *Salmonellae, Shigella, E.coli, Cryptosporidium*, and *Giardia.* In addition viruses can also cause enteric disease and every year these pathogens cause disease leading to hospitalisation for example Norovirus can cause severe disease requiring intensive treatment (Sakai et al., 2001). Routine laboratory diagnosis of these pathogens is carried out and recorded in an attempt to monitor the spread of these infections and target research and control priorities effectively; in addition the monitoring of these pathogens may lead to the early detection of new presentations of disease or novel diseases.

The importance of enteric diseases can be from the severity of diseases in an individual patient, from the number of cases involved in an outbreak or from both the severity and number of cases involved. The most common of these diseases in the UK are the viral pathogens. According to the Health Protection Agency for England the most commonly encountered bacterial cause of food borne infections are *Campylobacter* spp. Figures from Health Protection Scotland, the competent authority in Scotland, suggest *Campylobacter* associated disease in Scotland occurs at a rate of approximately 90 cases per 100,000 of the population making this numerically an important cause of enteric disease. This over all figure masks large ranges within health boards across the country as various

areas report markedly different rates. This variation may be due to a number of factors but the low population means that significant proportions are likely to be involved in outbreaks of the disease and confounding factors may be mistaken for correlation or causative factors.

Salmonellae species have reported infection rate of approximately 20 per 100,000 cases in Scotland and this has remained relatively stable with *S. enteriditis* being the major species reported. *S. typhimurium* is the second of the species noted as causing large numbers of laboratory isolates in Scotland.

Clostridium difficile is a growing concern as a cause of colitis amongst hospital patients in the UK; in Scotland there were approximately 5000 cases reported to the Health Protection Scotland service in 2005 (later totals unavailable on website) and this number has been steadily increasing for years. This is an important enteric disease however it is not typically food borne but is a common hospital acquired infection as it is associated with administration of broad spectrum antibiotics allowing overgrowth of this bacteria and subsequent overproduction of toxins. This bacterium can also cause the death of afflicted patients but unlike EHEC these are frequently already compromised in some fashion, by undergoing surgery for instance.

E. coli O157 is a less common cause of enteric disease and its incidence within the human population appears to be more variable and this appears to be related to meteorological conditions, most likely high rainfall, which may lead to increased faecal runoff from fields increasing the chance of human infection. The incidence of disease for Scotland overall was 4.8 cases per 100,000 however this hides a large variability within the health boards of the country. However these are relatively small populations and such figures may be artefactual. Over all the incidence is thought to be 1/100000 in Europe and it has frequently been noted that EHEC is more prevalent in Scotland than in other parts of

the world. Within Scotland the more agricultural regions tend to have higher incidence of disease. A more intensive study to establish a positive correlation is required but it would seem to be an uncontroversial suggestion that increased cattle numbers within an area will increase environmental contamination and exposure of the population.

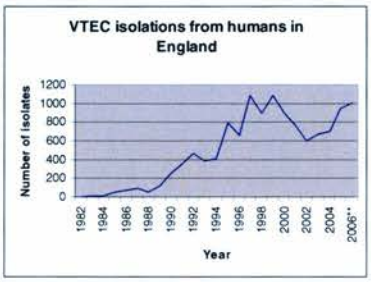
Shigella remains a relatively uncommon cause of gastroenteritis in Scotland with total cases for all species remaining at approximately 100 per year. Of the 4 species commonly isolated (*Sh. boydi, Sh dysenteriae, Sh flexneri, Sh. sonnei) S. sonnei* appears to be the most frequently isolated, followed by *Sh. flexneri* and *boydi.* Although causing approximately half the number of infections as EHEC it shares some pathological similarities with EHEC and although few in number the consequences of *Shigella* infection are potentially serious.

Bacteria are not the only causes of enteric infections in humans. Viruses and protozoa also have the potential to cause enteritis. Norovirus, sometimes called Norwalk virus, is frequently identified in Scotland as a cause of vomiting. (45.6 per 100000) and Rotavirus causes a similar level of infection in Scotland. *Cryptosporidium* also causes enteritis with some 400 cases reported to the HPS in 2006, with outbreaks occurring variably across the years. Another protozoon, *Giardia*, has also historically caused enteritis sporadically although some years may have no reports at all although underreporting is suspected due to a lack of standardisation between laboratories.

Figure 1 shows the number of EHEC and Salmonella infections reported in Scotland and England. Numerically EHEC is not a major cause of enteritis in humans however unlike all but *Shigella* this bacterium has high potential for fatalities resulting from extra-intestinal sequalae such as HUS. In Europe EHEC is thought to be the most prevalent cause of HUS with an estimated 85% of such cases in Germany being linked to EHEC infection (Bitzan et al., 1991; Bitzan et al., 1993). Although other enteric bacterial diseases can cause

mortality, such as *Clostridium difficile* these tend to be in compromised patients rather than the community-acquired infection of otherwise healthy individuals as can occur with EHEC.

Table a



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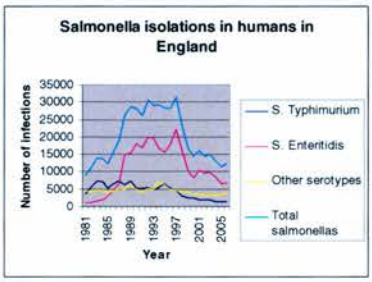

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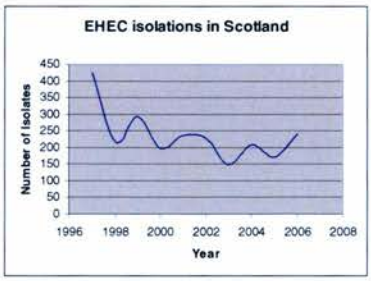

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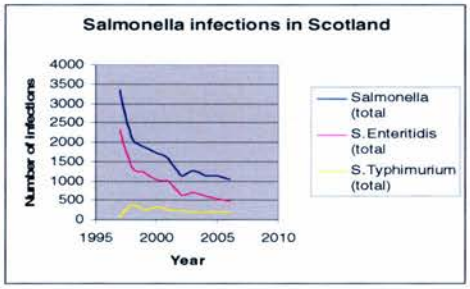

Figure 1 Graphs of bacterial infections in Scotland and England

The graphs are drawn from data published by Health protection Scotland and the Health protection agency England. Salmonellae and E. coli O157 strains are compared over the years 1997 -2006 for each. Table a shows VTEC isolations from England, B Salmonella infections from England. Tables c and d show data from Scotland.

Four outbreaks of *E .coli* O157:H7 associated disease occurred independently over the period January 06 to June 06 in the UK, 3 involving nursery schools. The total number of cases in the largest outbreak was 28 nursery age children and this resulted in 300 people requiring testing during the investigation of the outbreak. Investigation of an outbreak of this size requires intensive laboratory support and skilled knowledgeable workers available to investigate and carry out appropriate testing this involves a considerable amount of money to complete. The cost of lost working days for parents of children attending the nursery and similar losses of revenues are rarely quantified and may be a large factor in the overall cost of

an outbreak. In the cases of the nursery outbreaks no single source was identified to the public however in an outbreak in the Lanarkshire village of Law the local butcher was implicated as the source of contaminated foodstuffs. The premises were closed for the duration of the investigation. Further premises linked with the butchers were also closed and this represents a loss of revenue for the owner as potentially contaminated stock is tested, destroyed or otherwise rendered unsaleable while testing occurs. Once an establishment like a butcher is confirmed as a source of an outbreak the food chain above and below this point require to be investigated to establish the full extent of the contamination and where the bacteria entered the food chain.

A profitable farming business requires that a safe consumable product be produced. A loss of consumer confidence in the farming process will result in a crash in the price of the product being sold. The BSE (bovine spongiform encephalopathy) crisis and the FMDV (Foot and Mouth disease virus) outbreak were closely followed by the collapse of prices for meat and meat products. Carcase prices are critical in the farming community, the collapse of the carcase price pushed many of the farms to the brink in terms of economic viability and many businesses were unable to continue. Consumer confidence in the product during the BSE epidemic was lost and is another cause of economic loss that is rarely quantified.

In this light it is possible that mandatory testing for these diseases may be called for by the general public with farms required to certify that animals and animal products are pathogen free. As many other products on the farm use cattle by-products this may also include salad products from crops that are fertilised with animal manure. Health certification for this is likely to add costs to the final product placed on the shelf as this will require testing and an associated audit trail. At the current time there is a "Quality- assured" scheme in the UK which provides consumer assurance on issues such as welfare animal disease and

traceability of meat. Schemes similar to that run by QMS (Quality Meat Scotland) are found worldwide and as yet none of these zoonotic diseases are explicitly regulated by these organisations. As consumer awareness increases about the role of the farm in the occurrence of the disease within the human population demand for a zoonosis free component of schemes is certainly a real possibility. Due to the nature of bacterial enteric diseases continuous assessment would be required to maintain any assurance of freedom from these zoonotic pathogens as they are frequently asymptomatic in adult cattle.

Currently the most effective means of keeping the food chain free from contamination by zoonotic pathogens is application of the HACCP (Hazard analysis and critical control points) principles, and this is also a technique for continuous assessment of disease or pathogen status. In very basic terms this is the analysis of a production chain and the identification of points where the entry or replication of contaminants can be controlled. These may be points that require the food to be heated to a temperature that will render all organisms non-viable for example by cooking and HACCP identifies theses points where control measures can be introduced. For faecally carried organisms that are possible contaminants a critical point is the removal of the intestines from the carcase. Correct procedures will minimise the contamination of carcases and prevent spread throughout the rest of the meat being produced. Bacterial culture is not a routine component of HACCP protocols as the time lag of 24-48 hours to gain a positive result means that potentially contaminating bacteria can spread far and wide in the intervening period and very small numbers of bacteria may be able to initiate an infection; therefore very small amounts of material can spread over a great deal of any area where food production occurs and because of this HACCP will remain the most important method of ensuring food safety in the

slaughterhouse and in the food chain in general for some time yet. For those pathogens that are at very small levels in the carcase however the events on farm may be the most critical.

The farm is where all food production begins and the birth of the animal is the first point of contact where it can be exposed to zoonotic pathogens of the gut. The dam may be colonised symptomatically or asymptomatically by pathogens and pass these onto the young by direct contact, or through faecal contamination of the animals' environment. Normal gut development and function is dependent on the interaction of commensal flora and neonate intestine (Hooper et al., 2001), this usually occurs within 30 minutes of birth. In the normal environment it is impossible to stop this from occurring (except in specialised environments e.g. gnotobiotic environments). However eliminating certain pathogens may be possible on farm.

Detection of carriers of these bacteria would allow intervention measures to be carried out. This is problematic in the case of *E.coli* O157:H7 due to transient nature of colonisation in some cattle in which bacteria are detectable only for short periods of time and in low numbers, while other cattle may be what have been termed super-shedder (Matthews et al., 2006). The significance of these animals in herd colonisation dynamics is unclear currently but as these animals can shed tens of billions of bacteria it is likely to be important as removal of the single or minority of animals that are super shedders may well remove the infection from that farm. The detection of these animals is difficult as they display no overt signs of colonisation. Therefore whole herd testing is required to find possibly a single colonised animal. Once identified the animal could be removed, however in the intervening period a number of events are possible, the animal will almost certainly have passed on the bacteria to another animal, it could have lost the bacteria and no longer pose a

threat. In both cases the delay between sampling and delivery of test results is unacceptably long and obviates the rationale for testing.

Although *E. coli* O157:H7 is numerically a minor cause of enteric disease the serious nature of the disease and the considerable potential cost of investigation following outbreak give it considerably more importance than the number of cases suggests. There is considerable economic impact to outbreaks and these may have potential fatalities associated with these outbreaks. In addition the animal reservoir host means that there must be control measures in place at the slaughter plant and throughout the food chain.

EHEC is probably the most well known *E. coli* even though it is not the only disease associated with this group of organisms. Different *E. coli* have different pathological mechanisms that cause similar symptoms in humans, and many of those *E.coli* that cause disease in animals also cause diarrhoea the similarities and differences in pathological mechanisms and the bacterial influence on the host may allow insight into the role of EHEC in its reservoir host.

1.2 Escherichia coli

E. coli is a Gram negative bacterium which displays motility and is a facultative anaerobe. A member of the genus *Escherichia*, it is found in the gut of mammalian species and comprises a group of varied organisms that have specialised in a number of ways to exploit this environment; other members of this genus are not considered medically important. Colonisation of this environment occurs rapidly following birth and bacteria can be isolated from the neonate gut very quickly (Ducluzeau, 1993). The group includes members that are traditionally considered as commensal organisms, causing no apparent harm to the host however some strains of bacteria which are capable of inducing pathological responses may be acquired at the same time (Peter et al., 1999) but there appears to be no association of disease with a specific pathogen in infants. The host species may also be important as bacteria of a variety of species can be carried by a host species without ill effect but transmitted to another host may be able to cause severe consequences. Although normally contained within the intestinal tract the bacteria can escape from this location. In these cases normally commensal *E coli* may be able to cause disease. Compromised hosts (those with immune dysfunction or implants) show impaired ability to control normal flora, this may lead to pathologies associated with otherwise non-pathological bacteria.

1.3 Identification and classification of *E.coli*

1.3.1 Biochemical identification.

These bacteria can be grown on a number of substrates including LB agar, mackonkey agar which allow selective growth of the bacteria. Bacterial growth at 37 °C is frequently enough to allow identification and morphology of the colonies grown is, along with specific indicator media, frequently enough to allow presumptive identification of the pathogen. In addition there are commercially available biochemical test strips that detect metabolic activities of the bacteria and that can be used to further characterise the bacterium (Dennstedt et al., 1983; Swanson and Collins, 1980).

1.3.2 Serological identification.

Another approach that has been used to identify *E. coli* is the use of serology. *E. coli* have a variety of surface components of which 4 have been used routinely to allow identification and grouping of these bacteria. Antibodies raised against these components can be used to identify and classify the bacteria. These 4 antigens are: the somatic O-antigen, the K- antigen, the H-antigens and F-antigens and this has been used in the investigation of *E. coli* found in various species (Rippinger et al., 1995; Miyata et al., 1994) as shown in Figure 2.

O-antigens are one of three components of lipopolysaccharide and compromise repeating sugar moieties linked via a core oligosaccharide to lipid A in the outer leaflet of the bacterial outer membrane. The 3 remaining antigens are surface complexes of heterogeneous nature with K-antigens being capsular polysaccharides and F-antigens being proteins forming fimbrial adhesins. H-antigens are the flagellum of the bacterium which consists of

multimeric units of the protein, flagella, forming a helix. This structure is attached to the hook a flexible joining structure attaching the filament to the basal body which provides motive power to the filament and the bacterium as a whole (DePamphilis and Adler, 1971). Usually O and H antigens are paired to make a serotype, and this format has been used to identify sub-species groups of bacteria and establish relationships.

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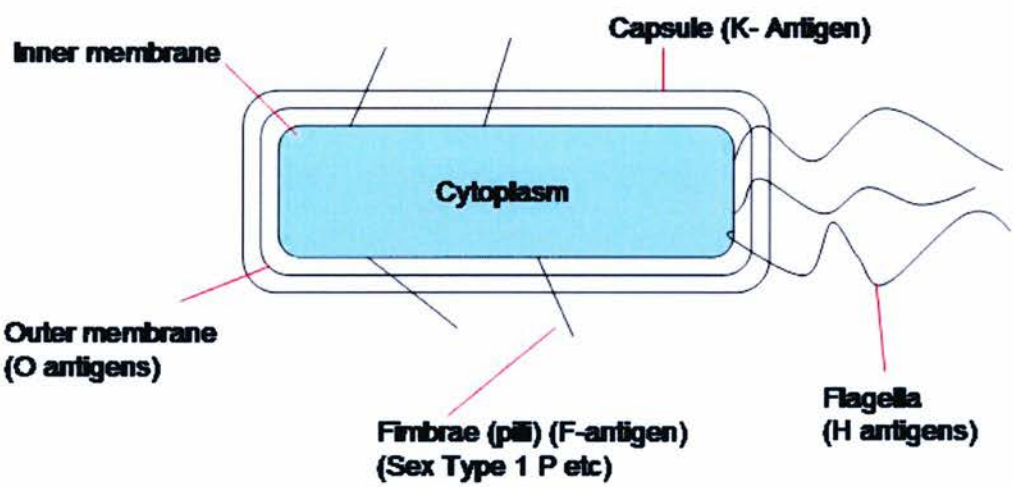


Figure 2. Structures of E.coli. E. coli bacterium showing the major structures that compose its structure.

The major typing antigens are shown and include the flagellum (H), capsule (K), the fimbriae (F) of various types, and LPS, the O antigen

1.4 Pathotypes of *E.coli.*

E. coli pathotypes are groupings of bacteria that share similar virulence factors and due to these factors similar pathologies are associated with colonisation by bacteria in *E. coli* pathotypes. To date 5 intestinal and 2 extra-intestinal pathotypes of pathogenic *E.coli* have been described.

Intestinal *E. coli* Pathotypes:-

1. Enterotoxigenic E. *coli.* (ETEC).
2. Enteroaggregative *E. coli* (EAEC).
3. Enteroinvasive *E. coli* (EIAC)
4. Enteropathogenic *E. coli* (EPEC).
5. Enterohaemorrhagic *E. coli* (EHEC)

Extra-intestinal *E. coli* Pathotypes.

1. Urinary infection associated *E. coli* (UPEC)
2. Neonatal Meningitis associated *E.coli* (NMEC).

1.4.1. Enterotoxigenic E.coli (ETEC)

Enterotoxigenic *E. coli* (ETEC) causes major disease in young children in the developing world and is the suspected causative agent in travellers' diarrhoea. It is also a cause of disease in neonatal piglets and cattle (Pohl et al., 1989; Monckton and Hasse, 1988; Nagy and Fekete, 1999). The primary source of bacteria is from contaminated water and food (Qadri et al., 2005).

The pathology of ETEC associated disease is caused by the elaboration of toxins. These toxins are at least one of two types, the heat labile toxins (LT) or the heat stable toxins (ST) and ETEC may carry one or both of these toxins. LTs is an AB_5 subunit toxin type in which the toxin is endocytosed and translocated within the cell where the A-subunit is able to transfer an ATP- ribosyl moiety to the GTP binding protein, Gs (Nataro and Kaper, 1998; Moss et al., 1979). This leads to the over-activation of adenylate cyclase with the resultant increase in the levels of cAMP, activation of chloride ion channels is increased, particularly the Cystic fibrosis transmembrane receptor (CFTR) channel. This chloride ion channel pumps chloride into the lumen (Clarke and Harline, 1996). The movement of this ion into the lumen is one of many linked channels that allow control of fluid across the epithelium.

STs are toxins consisting of a chain of 18-19 amino acids that bind an apically located receptor, guanylate cyclase. This causes an elevation in the levels of intracellular cGMP and activation of the CFTR channel in a manner similar to the LT toxin resulting in the loss of electrolytes (bicarbonate and chloride) into the lumen of the intestine and resulting in an osmotic gradient that leads to watery diarrhoea. A second ST, STb is found in pig isolates (Kaper et al., 2004). This causes gross damage to the villi of the intestine along with secretion of bicarbonate. The mechanism by which the STb toxin causes diarrhoea is as yet undescribed as is the receptor to which it binds.

Both of these pathological mechanisms result in watery diarrhoea due to osmosis thus loss of fluid and electrolyte imbalances are a major component in these diseases.

1.4.2. Enteroaggregative E.coli (EAEC)

Enteroaggregative E.coli (EAEC) causes persistent and watery diarrhoea in both infants and adults. The disease associated with these bacteria is usually extended sometimes taking over 14 days to resolve. Infections are usually sporadic and endemic in nature however epidemic outbreaks have been noted. These bacteria are common in the developing world but are less commonly noted in the developed world including the UK and Europe.

EAEC attach to the intestinal wall of affected patients in a distinctive pattern, histological examination of the mucosa reveals bacteria in a brick wall like arrangement. In colonised intestine mucus secretion is enhanced and this is combined with shortening of the villi, haemorrhagic necrosis and subsequent mucosal destruction. These pathological effects may be a result of several toxins though the relevance of any particular toxin is unknown (Law and Chart, 1998). The result of this is an impairment of absorption and secretory physiological functions in the intestine. This inability to control fluid loss leads to diarrhoea as electrolytes can cross the epithelial barrier in an uncontrolled fashion but the full pathology of this pathotype is as yet unclear.

Another subset of this pathotype is Diffusely Aggregative *E. coli* (DAEC), a related bacterium. It is associated with disease in slightly older children (1-4 years of age) but has not been reported outside this cohort. It adheres in a slightly different pattern to EAEC in the Hep-2 adherence assay (Beutin et al., 2003; Scaletsky et al., 1999). It is reported to cause watery diarrhoea and there appears some evidence for inflammatory responses by DAEC (Meraz et al., 2006; Arikawa et al., 2005; Meraz et al., 2006) although once again the requirement for specific virulence factors is unknown.

1.4.3. Enteroinvasive *E.coli* (EIEC)

Enteroinvasive *E. coli* is implicated in epidemics of watery diarrhoea that may be indistinguishable clinically from the diarrhoea caused by other *E. coli* and *Shigella*. The similarity between this bacterium and *Shigella* suggests that it is possible that these are identified incorrectly as two differing species. Transmission is thought to be from contaminated food and water and this bacterium is therefore prevalent in places where water sanitation can be expected to be poor; consequently it is less prevalent in the developed world. EIEC shares epidemiological similarities in this respect to *Shigella* which it resembles in many aspects. However the infective dose appears to be larger than that of *Shigella* therefore there is less likelihood for person to person transmission.

The ability to invade epithelial cells is shared by Enteroinvasive *E.coli* and *Shigella* species, both EIEC and *Shigella* are able to enter the cytoplasm of cells following invasion of the cells and lysis of the endoplasmic vacuoles. The genes required for this are carried on a plasmid, pInv, in *Shigella* and similar genes are found in EIEC. Both bacterial species invade the epithelium and once in the cytoplasm can move and replicate intracellularly. This infection is associated with an inflammatory response and this is characterised histologically as ulceration of the colonic mucosa. The damaged mucosa is unable to control the fluid and electrolyte balance within the gut.

1.4.4. Enteropathogenic E.coli (EPEC).

Enteropathogenic *E. coli* (EPEC) are a common cause of diarrhoea in a many parts of the world in children, birds, farm animals and pets (Okerman, 1987; Goffaux et al., 2000; Pakpinyo et al., 2002).

The defining characteristic of this disease causing bacterium is the appearance of characteristic lesions in the epithelial cells of infected animals and patients. These are the attaching and effacing (A/E) lesions and the bacterial determinants responsible for this lesion as well as pathophysiological processes will be discussed in more detail as part of the EHEC bacterium as it is shared with EHEC. These lesions are able to reduce the absorptive area and alter the physiological function of epithelial cells. The dysregulation of fluid control gives rise to the clinical signs of disease which like all other pathotypes is primarily diarrhoea.

1.4.5 VTEC/STEC.

In 1983 a new syndrome associated was noted by investigators (Riley et al., 1983; Karmali et al., 1983) as diarrhoea was accompanied by specific group of clinical signs including haemorrhagic colitis (HC) a bloody diarrhoea associated with little or no fever, haemolytic ureamic syndrome (HUS), a renal condition defined by the presence of thrombocytopenia, microangiopathic haemolytic anaemia and acute renal failure. Investigators were able to identify a specific cytotoxin associated with this clinical presentation. This cytotoxin was associated with apoptosis in Vero cells unlike the action of already known toxins associated with other *E. coli* pathotypes such as the LT and ST toxins of ETEC. This toxin was subsequently identified as being related to a toxin found in *Shigella* species. Nomenclature for this toxin has been confused as the terms Shiga-like toxin and

Shiga-toxin are in common usage and both appear to be used interchangeably in the literature to date. This toxin was initially found in the serotype O157:H7 but has subsequently been found in other serotypes, and all serotypes including O157:H7 may be Shiga-toxin negative. The presence of shigatoxin alone is not in itself sufficient to confer pathogenicity and other virulence factors are required for *E. coli* to produce the HUS and HC associated with this clinical syndrome thus bacteria that carry shigatoxin have come to be termed Shigatoxigenic/Verotoxigenic *E. coli* (VTEC/STEC).

1.4.6. Enterohaemorrhagic *E. coli* (EHEC)

EHEC are a subset of the STEC group of bacterium which possess the LEE island as well as Stx. Although the first *E. coli* serotype identified as carrying the genes for shigatoxin, *E.coli* O157:H7 is not the only serotype that carries these however pathogenicity of EHEC also requires the carriage of the LEE pathogenicity island. As mentioned previously, this genomic region is also found in the EPEC pathotype, and this encodes the proteins that are involved in the production in the attaching and effacing lesions seen histologically within the intestine of EPEC affected patients. *E. coli* serotypes that have both the LEE Pathogenicity island within the genome and shigatoxin are referred to as Enterohaemorrhagic *E. coli* (EHEC).

1.5 Virulence factors of EHEC

1.5.1 Shiga-like Toxin (Stx)

The most severe pathology of EHEC disease is caused by the toxin Stx. Pathology is described grossly as Haemorrhagic colitis (HC), and haemolytic ureamic syndrome (HUS) which has its three syndromes of renal failure, thrombocytopenia and microangiopathic haemolytic anaemia. The histological lesion associated with each of these pathologies is apoptosis of specific cellular subsets within the intestine or the renal system. Cells lining the endothelium of intestinal blood vessels are bound by the toxin and apoptosis is induced and this leads to the blood seen in the faeces of afflicted patients as integrity of the blood vessels within the intestine is lost. Within the renal system the endothelium of the glomeruli are also intoxicated with apoptosis being induced in these cells (Pijpers et al., 2001) and blood loss occurs as a result of the loss of integrity in the blood vessels associated with the kidney. The transfer of the toxin to the renal system from the intestine is still speculative however internalisation by neutrophils has been suggested (te Loo et al., 2000) with subsequent transfer to human renal endothelial cells. Stx is an A_1B_5 subunit type, with the A subunit linked to a B-subunit pentamer. The toxin is encoded on mobile genetic elements, specifically bacteriophages that are considered to have been acquired from *Shigella dysenteriae* as it retains a significant homology with the toxin (Shiga toxin) found within this species (Nakao and Takeda, 2000).

The receptor for Stx is the glycolipid Gb3, (Globotriaosylceramide) (Nyholm et al., 1995; Okuda et al., 2006) although another glycolipid subtype, Gb4 (Globotetracylceramide), is also thought to bind the B subunits of Shiga-toxin and perhaps allow toxin specificity (Degrandis et al., 1989). Toxin uptake into the cells is guided by receptor-mediated endocytosis (Lord et al., 1999) Gb3 binds Stx on the cell surface via the B subunits of the

toxin. The intracellular domain of Gb3 and associated proteins recruits clathrin along with accessory proteins to the area of membrane below the receptors initially forming a pit in the cell surface. The clathrin triskelion builds a polyhedron structure and allows formation of endosomes (Shen and Turner, 2005). This receptor-mediated endocytosis is shown as a diagram (Figure 4). Intracellular trafficking of these endosomes allows retrograde transport of Stx into the endoplasmic reticulum. Subsequent to this A subunit targeting and cleavage of the 28S rRNA of the Ribosome blocks the elongation step of protein synthesis and leads to the cellular apoptosis (Lingwood, 1996) seen in both the renal system and intestine (Shown figure 3). The acylation of Gb3 receptor fatty acid (acyl) tails embedded within the cellular membrane plays a role in the outcome of disease. The longer acyl tails of Gb3 receptors in cattle epithelium may be important in mediating trafficking to lysosomes (Lingwood et al., 1998) (Hoey et al., 2003) and this may be important in the eventual host response to Shiga-toxin intoxication. Stx activity is a complex area which is a composite of both the receptor and the toxin itself and although in human infections it is classically regarded as a toxin there may be effects and activities that are undefined. This may be important in the ruminant host where toxic activity is not seen although the receptor is expressed and bound in the epithelium of colonised animals.

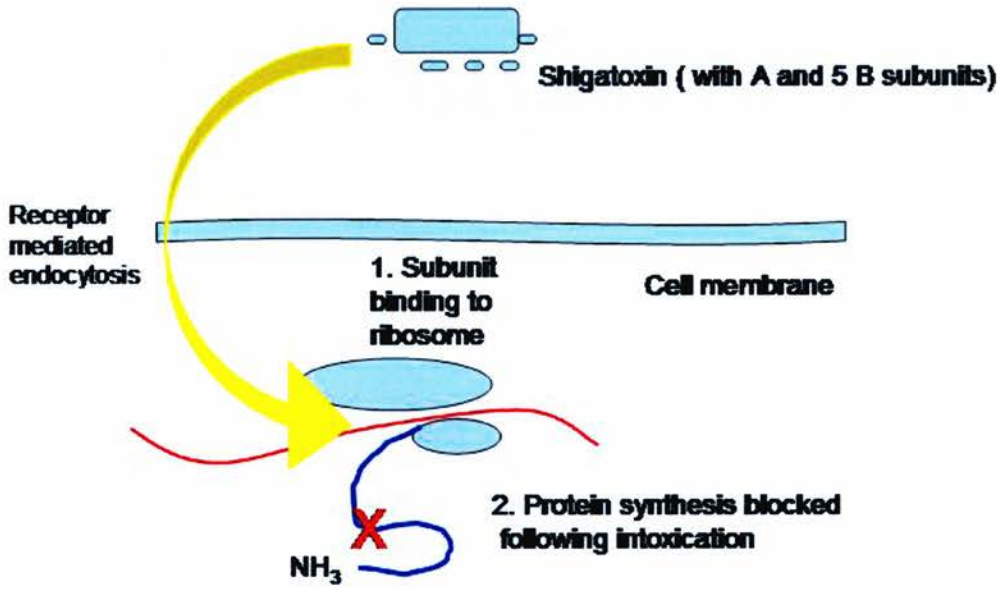


Figure 3. Shigatoxin activity.

Stx Inhibition of protein synthesis follows internalisation of the A subunit. This binds to the 28s ribosome subunit following intracellular trafficking and cleaves ribosomal RNA at a specific residue. Cleavage by the A subunit stops elongation of the protein and hence synthesis of the whole protein.

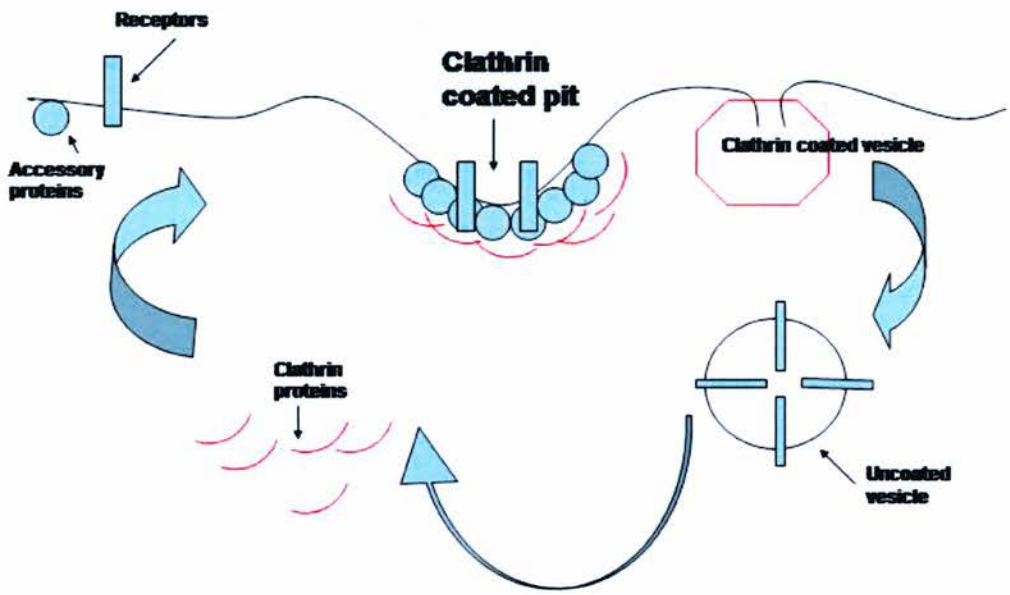


Figure 4. Clathrin mediated endocytosis

Following binding of receptors by their ligands accessory molecules (AP2) bind to the intracellular domain of the receptor. This in turn mediates binding of the clathrin triskelion. The structure of these molecules induces formation of a pit and then a coated vesicle.

1.5.2 LEE Pathogenicity Island.

This is a region of the bacterial genome that encodes all the genes required for the production of an attaching and effacing lesion. Due to the different G+C content of this portion of the genome compared to the "core" *E. coli* genome, this is thought to have been acquired from another species although its origin is obscure (Sperandio et al., 1998). In EPEC and EHEC the important proteins used to produce A/E lesions are the type three-secretion system which is composed of Esp proteins, intimin and TIR (de Grado M. et al., 1999). These proteins function collectively to produce the attaching and effacing lesions seen histologically in clinical cases of both EPEC and EHEC infection.

The LEE portion of the genome encodes a type three secretory system (TTSS), the system can be visualised as a molecular syringe and Needle. The structure of the TTSS is composed of a number of proteins and the LEE is subdivided into sections (LEE1-5), the basal components are encoded on LEE1-3 and Ler is also found here which acts as a regulator for further LEE proteins (Elliott et al., 2000). The four Esp proteins A, B, D and EscN are membrane or surface components of *E. coli*, (Jarvis et al., 1995). The exact mechanisms are not assigned to each protein but A is thought to form a filament (Wilson et al., 2001) which allows other bacterial proteins to be translocated, while EscN is a true inner membrane ATPase which drives the transport (Gauthier and Finlay, 2003). EspB and D form pores in the host cell membrane to allow *E. coli* effector proteins to enter the host cell (Vallance and Finlay, 2000). A diagrammatic representation is shown in Figure 5.

Encoded on the LEE5 region of this Pathogenicity Island are Intimin and the Translocated Intimin Receptor (TIR). TIR is secreted into the host as an effector protein into the cytoplasm of the host cells. This allows Tir to be inserted into the apical cell

membrane of the host cells and this is a critical event in the formation of the attaching and effacing lesions. Although conserved between EHEC and EPEC, there are some differences between the formation of the attaching and effacing lesions and in the case of EHEC as phosphorylation occurs in EPEC but not EHEC Tir the phosphorylation step is not required before the insertion of TIR into the host cell membrane (DeVinney et al., 1999). However full pathogenicity by EHEC requires factors that are encoded elsewhere in the EHEC genome (Campellone et al., 2004) such as EspFu. Therefore there are some differences between EPEC and EHEC molecular pathology which may reflect further differences between these two pathogens that have yet to be determined.

Intimin is another LEE5 bacterial protein that remains associated with the bacterial surface. It is encoded on the LEE Island and there is a significant heterogeneity of structure within these proteins throughout the serotypes of EPEC and EHEC (Ramachandran et al., 2003) which allows intestinal tropism (Fitzhenry et al., 2002; Reece et al., 2001). Intimin is retained in the outer membrane of the bacterium and interacts with Tir which is embedded within the host cell. The binding of Intimin to Tir allows intimate attachment of the bacteria to the host and initiates further changes within the host cell (see Figure 5).

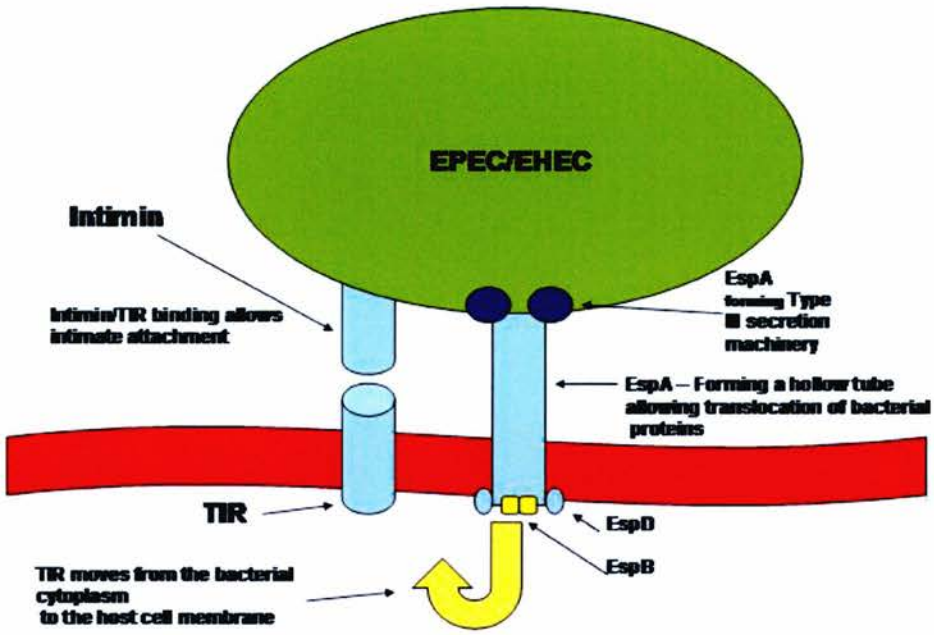


Figure 5. The type three secretion system of EPEC and EHEC.

This shows the arrangement of the Type three secretion system ad its association with the host cell. TIR is translocated into the host cell to allow intimate adherence by the bacterium.

EHEC forms an intimate attachment as a consequence of intimin/Tir binding with the epithelial cell membrane and an accumulation of actin in the cell below the bacteria can be seen. A number of secreted factors are involved in the changes in actin within the cell which include actin accumulation below the bacteria (Goosney et al., 2000) and these changes are mediated by a number of factors produced by *E. coli* including EspG (Hardwidge et al., 2005; Tomson et al., 2005) which may alter tight junctions of the cell and contribute to barrier disruption and map (mitochondrial associated protein) which may also

influence tight junctions (Ma et al., 2006). Similar lesions are seen on the mouse model of the A/E bacteria *Citrobacter rodentium* (Deng et al., 2003). The resultant A/E lesions result in the loss of these microvilli which has the effect of reducing the surface area of the intestinal epithelial cells and may be one reason for the onset of diarrhoea in clinical cases.

1.5.3 Flagella

The flagellum is an organelle required for motility of the bacteria. It provides motive force to allow directed movement along chemotactic gradients. Flagella are major pro-inflammatory ligands in the human (Hayashi et al., 2001) and flagellin of many species including *E. coli* and *Salmonella* are sufficient to cause an inflammatory response (Eaves-Pyles et al., 2001). Flagella are closely related to the TTSS sharing significant homology with this structure and the spindle and motor imparts a wave-like motion to the flagella.

Inflammation occurs when the bacteria contact the epithelium and the flagellar protein makes contact with the cell membrane and its surface proteins. Flagella are detected by Toll-like receptor 5 (TLR5) a member of the Toll family of proteins (Hayashi et al., 2001). These proteins form part of the innate immune system and detect a variety of bacterial components. These receptors bind bacterial components through the leucine rich repeats. The intracellular domain of the Toll receptors interact with Myeloid differentiation factor 88 (MyD88) and Il-1 receptor-associated kinases (IRAK) (Medzhitov et al., 1998; Muzio et al., 1997) which leads to the activation of NF-κB (Zhou et al., 2003). TLR5 is linked to the adaptor protein MyD88 that phosphorylates further proteins that include IRAK family proteins. Once IRAK has undergone phosphorylation recruitment of TRAF-6 (tumour necrosis receptor- associated factor 6) which can phosphorylate the transcription

factor inhibitor IκB which binds NF-κB, although MAP kinases can also be activated (Akira and Hoshino, 2003). Following IκB is phosphorylation NF-κB is released allowing it to translocate from the cytosol into the nucleus. NF-κB is a DNA binding protein that binds to specific motifs within the cellular DNA and this allows the transcription of targeted genes. Once in the nucleus NF-κB acts as to promote IL-8 expression (amongst other genes) to induce an inflammatory response (Figure 6). As already stated binding of Flagella is through the extracellular portion of the TLR5 which has a leucine rich repeat region and it is this portion which is thought to mediate binding to pathogen components.

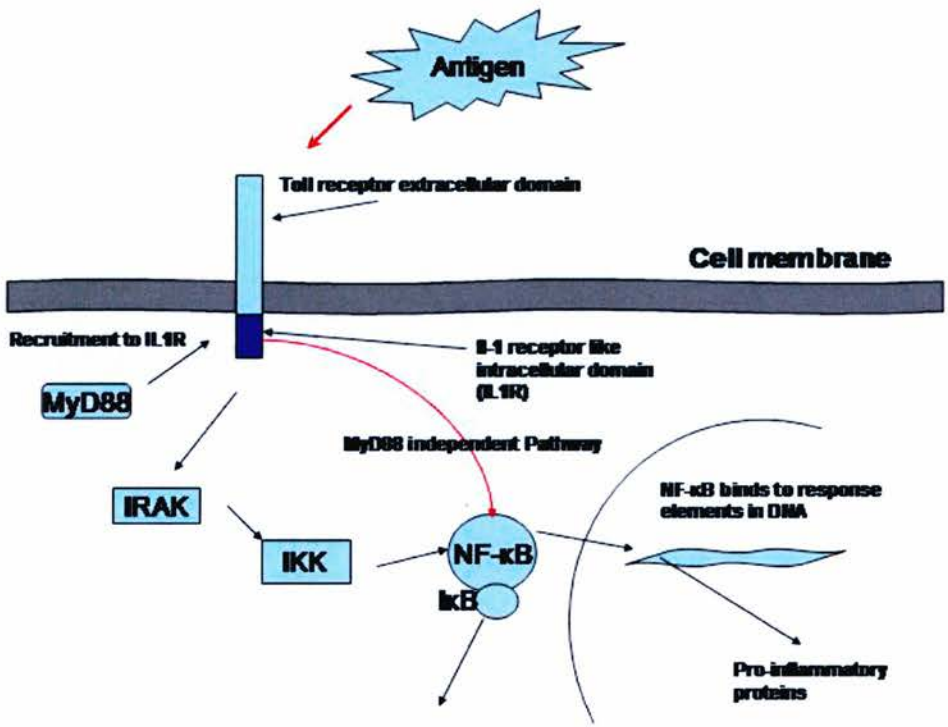


Figure 6. Outline scheme of TLR pathways.

Toll like receptors on the cell surface bind there ligands and recruit proteins including MyD88 to the intracellular domain. This initiates a kinase cascade that results in Nf-κB to DNA and transcription of a variety of factors. MyD88 independent pathways also exist and allow alternative pathways for production of proinflammatory ligands.

1.5.4 Other EHEC secreted virulence factors.

There are other secreted factors in the genome of EHEC which have effects on the host cell. Map and EspG have been described as having effects of tight junctions and cell death, another that has effects on cell junctions is EspF (McNamara et al., 2001). EspF is also involved in cell death by apoptosis. EspL is another bacterial effector that is associated with increased severity of disease, the model organism *Citrobacter rodentium* displayed reduced pathogenicity without EspL (Mundy et al., 2004a; Bals, 2000; Mundy et al., 2004b), the specific effect in EHEC or EPEC disease is less clear. EspH is associated with attachment to the cell surface and bacteria deficient in EspH are less adherent to host cells but leave the pedestal formation in place (Shaw et al., 2005). There are likely to be further bacterial factors that have a virulence role remaining to be discovered.

Enterohemolysin is an RTX toxin related to similar toxins in other bacterial species (Pellett and Welch, 1996; Bauer and Welch, 1996a) and is able to induce pore formation in erythrocytes (Bauer and Welch, 1996b; Menestrina et al., 1994). Various functions have been proposed for this virulence factor including phagocyte destruction (Welch et al., 1995) or cytokine induction for monocytes (Taneike et al., 2002). The toxin may be retained to prevent phagocytosis of the bacterium and extend colonisation.

The pO157plasmid is widespread in human isolates of EHEC (Levine et al., 1987) and encodes ToxB. *E. coli* O157:H7 also contains a homologue of the Enteropathogenic virulence factor LifA termed Efa1' which is a truncated version of the Efa-1 found in non-O157 EHEC (Stevens et al., 2004) and although the full length version of Efa-1 is associated with the inhibition of lymphocyte proliferation and may mediate intestinal tropism neither the truncated version, Efa1', nor ToxB are associated with this effect (Abu-Median et al., 2006)

1.6 Pathogenesis of the disease in human cases

EHEC is not a commensal of the human gut nor is it found commonly in the environment. Infection of the human requires contamination by ruminant faeces. Usually this is a result of sub optimal slaughterhouse technique, but faecal contamination of the environment can lead to contaminated pasture, vegetables, and water. This is important as EHEC has a very low infectious dose, estimated at a few hundred infectious units (Alexandre and Prado, 2003; Bolton and Aird, 1998), approximately one hundredth that of other Bacteria that cause diarrhoea.

The pathogenesis of disease caused by EHEC involves several steps which are normally presented sequentially but may occur contemporaneously. These steps are:

1. initial binding
2. Type three secretion system activation
3. Tir/intimin binding
4. actin rearrangement
5. Neutrophil recruitment
6. Shiga-toxin intoxication

The bacterium must come into contact with the host cell to initiate pathogenesis and the structures involved in this are still to be identified, although a cell surface glycolipid asialoGM1 (ASGM1) (McNamara et al., 2006) is a possible candidate for this initial binding step allowing the bacteria to remain in close proximity to the cell membrane. ASGM1 is thought to be able to bind the flagellum of a variety of bacteria. This glycolipid does not appear to have transmembrane or cytosolic portions but ligation of the ASGM1 produces an increase in ATP within the cell and results in the activation of the ERK pathway. This has

been shown to up regulate mucin gene expression in response to flagella. The presence of mucin glycoprotein on the surface of the cell may be sufficient to interfere with binding of *E. coli* O157:H7 to the epithelium and StcE is thought to act as a mucinase to allow the bacterium to colonise the host (Grys et al., 2005; Grys et al., 2006). Pathogenesis requires close association of bacterial and eukaryotic cell membranes and although Flagella are important in the attachment it is not vital as aflagellate bacteria are capable of binding in vivo (Dobbin et al., 2006).

Close association of the bacterial and cell membranes allows the TTSS to bridge the gap and allows A/E lesion formation to occur. The TTSS is a molecular syringe that creates a connection between the cytosol of the bacteria and host cell, the associated bacterial protein EscN provides energy to drive the remainder of the bacterial proteins into the host. These proteins include TIR and the Esp proteins. Actin rearrangement then occurs in the area underneath the bacterium and the characteristic histopathological lesions are seen. This change in the actin structure is a result of the insertion of TIR into the cell membrane and its interaction with the bacterial membrane protein Intimin (de Grado M. et al., 1999). Actin is polymerised and attached to the intimin and staining shows an increased accumulation below the bacterium and this is characteristic of both EPEC and EHEC infections.

The rearrangement of actin alters the host cell cytoskeleton and this has effects within the cell and on the host cell cytoskeleton. Actin also interacts with other components in the cell including the tight junctions. Tight junctions are sometimes referred to as desmosomes and hemidesmosomes within the epithelium. These structures function to allow adherence between cells and the underlying matrix additionally these allow control of diffusion around the cell and in doing both of these things it maintains the integrity of

epithelium. Actin binds to the tight junctions internally within the cells and when A/E lesions occur the actin is rearranged and disperses from the junctions. This phenotype is associated with the EspF proteins of the LEE (Elliott et al., 2002). This results in a change in the distribution of tight junction proteins with these proteins being distributed throughout the cell rather than at the cell membrane. This disruption breaks the ability of the tight junction to maintain polarity of the cell (Canil et al., 1993) and basolateral proteins may move to the Apical surface (Muza-Moons et al., 2003). The ability to limit paracellular diffusion of osmotically important ions is also compromised contributing to the diarrhoea seen in human cases. This bacterial phenotype is likely to increase the spread of the bacterium as the volume of faeces is increased and it increases the likelihood of bacterial spread.

An important aspect of the pathogenesis of the EHEC associated disease is the inflammatory response that occurs. This is seen histologically as an increase in the neutrophils within the epithelium and an increase in the overall thickness of the intestine due to oedema at the site of colonisation. Much of the inflammatory response to EHEC is thought to be mediated by the TLR-5/Flagellin interaction via the NF-κB pathway. Although the interaction of flagella with ASGM1 has been discussed the interaction of Flagellin with TLR-5 is likely to be one of the most important in producing inflammatory responses by inducing the intracellular pathway that leads to NF-κB binding to DNA. The production of pro inflammatory chemokines follows transcription activation by NF-κB. IL-8, one of the chemokines induced by NF-κB is a chemo- attractant and is the major signal that brings neutrophils to the site of colonisation (Kucharzik et al., 2005). Although the flagellum is thought to be the major proinflammatory ligand other factors in the EHEC

genome may be pro inflammatory and aflagellate bacterium are also the cause of severe enteric disease (Beutin, 2006).

Another ligand, lipopolysaccharide (LPS) is also bound by TLR receptors specifically TLR-4 and this may also be involved in the inflammatory response to EHEC in addition to TLR-5 but LPS may have other roles and in fact may influence cellular proliferation amongst other aspects of physiology (Olaya et al., 1999). TLR-4 is also relatively under expressed in intestinal epithelial cells (Hoshino et al., 1999). Toll interacting protein (TOLLIP) is a protein involved in down regulating the response to TLR ligand interaction and interacts with IRAK as a negative regulator of its kinase activity, and this protein is relatively highly expressed in intestinal epithelial cells reducing the response to commensal Gram negative bacteria (Didierlaurent et al., 2006) (Melmed et al., 2003) and possibly avoiding inappropriate immunological responses to the resident bacteria.

In addition to IL-8 other host molecules are likely to be produced in response to colonisation by EHEC including CCL20/MIP3α. These responses will mediate pathogen clearance and tolerance through such methods as the production of antibacterial proteins or the inducation of secretory diarrhoea although the role of diarrhoea is unclear in the final outcome of disease. However the most noticeable effect in human disease associated with A/E pathogens is the attraction of neutrophils to the site of infection (Savkovic et al., 1996; Michail et al., 2003). Il-8 is a potent chemoattractant and as such it will attract the neutrophils to the sub epithelial space.

The intestinal epithelial cells normally form a barrier to most large molecules and particles including Shigatoxin but following epithelial disruption, mediated by both the functions of LEE discussed above and the action of neutrophils damaging the tight junctions, this barrier is impaired. Normal control of paracellular diffusion which requires

intact tight junctions is lost. As noted previously, Stx is a major determinant of human disease and epithelial disruption promotes transfer of this toxin from the lumen to the tissues. Once across the basement membrane of the gut epithelium toxin is free to bind to any Gb3 positive cells available and in the gut the endothelium of the microvasculature expresses Gb3 and binding by Stx allows internalisation of the cell and blocking of protein synthesis at the ribosome. The loss of endothelial cells due to necrosis means that the blood vessels lose integrity and bleeding occurs into the intestine, Control of fluid within the circulatory system is dependant on an intact endothelium (Bielaszewska and Karch, 2005). Red blood cells and fluid can then leave the circulatory system. The blood can then pass into the lumen of the gut due to the loss of tight junctions in the gut as previously described and presents clinically as Heamorraghic colitis.

Further transport of the toxin can occur either through the movement free in the circulatory system, or through the movement of neutrophils within tissues as Stx is able to bind receptors on neutrophils. Although at a lower level than Gb3 binding neutrophil binding of Stx allows movement in the circulatory system and this results in the dissemination of the toxin (te Loo et al., 2000; Melmed et al., 2003). Incubation of neutrophils loaded with toxin has been demonstrated to cause inhibition of protein synthesis and cell death. In the renal system bleeding into the urine occurs as a result of this damage to the glomeruli and this blood loss is seen as bloody urine. Although the loss of the cells is due in the main to toxin action, there is evidence of Il-8, IL-1β and TNF-α at the infected kidneys and macrophage activation may have a role in this (Nakao and Takeda, 2000). The damage to the renal system results in renal failure with total loss of control of urine with potentially dehydration occurring as fluid is lost.

1.7 EHEC in cattle.

EHEC infection in humans usually results from contamination of the food chain or water courses with faeces from cattle. Cattle may be colonised and shed large numbers of bacteria per gramme of faeces with some animals contributing a disproportionately large amount of the contamination – so-called "super-shedders" (Matthews et al., 2006) - however such a colonisation may only occur for approximately 7 days (Liu et al., 2005) and without clinical signs being apparent in all but a very small minority of cases. Very large numbers of bacteria in the faeces of individuals may reflect transmission dynamics rather than underlying differences in cattle genetics that may predispose certain animals to colonisation and it has not been conclusively shown whether the super-shedder hypothesis holds generally.

The site of colonisation in cattle is the terminal rectum, and particularly the follicle associated epithelium in this location. In the field situation there are no consistent reports of immunologically competent animals (Adults and calves with sufficient colostral intake) showing clinical symptoms including diarrhoea but there is pathology at the histological level when the colonisation site, the terminal rectum, is examined (Naylor et al., 2003). The presence of attaching and effacing lesions on bovine intestinal epithelial cells is noted (Naylor et al., 2005; Dean-Nystrom et al., 1997). Colonies occur at various points along the intestinal tract including the colon but predominantly occur at the recto-anal junction especially in longstanding colonisation. It is suggested that in calves less than 36 hours old diarrhoea can be induced however many bacteria are able to induce disease in this age cohort of cattle (Barrington et al., 2002; Acres et al., 1975). The relevance of this observation to the biology of the EHEC in the field situation is doubtful. Bovine neonatal diarrhoea is a complex syndrome which is the result of multiple aetiologies and the influence

of a single aetiology can be difficult to discern. The attaching and effacing lesions allow intimate attachment to the epithelium and this may allow prolonged colonisation compared to the luminal contents and a prolonged time for the bacterium to be passed out in the faeces. However there is little evidence of there being an immunological response, a major difference between the bovine response and the pathological response in the human to O157 serotypes. Studies have suggested that other serotypes are able to induce a response by mechanisms that are not yet known and there may be a shift in intraepithelial lymphocytes away from the CD8+(alpha) subtype caused by colonisation of EHEC serotypes (Menge et al., 2004).

Shiga-toxin is not in the case of cattle an inert protein that appears to have no effect on the epithelial cells of the bovine intestine. Shiga-toxin receptors are expressed in the intestinal epithelium and specifically the epithelial cells that are exposed to the contents of the intestine (Hoey et al., 2002) trafficking of the toxin within these cells differs from that in the human. Toxin appears to be sequestered away from the ribosomes of cells where protein elongation block occur in the human and towards lysosomes where any further function that may occur in the bovine is unknown. However in vitro experiments suggest that Shiga-toxin is able to reduce the activation and proliferation of lymphocyte subsets (Menge et al., 1999). This may induce a reduction in the adaptive immune response to STEC or EHEC at the bovine mucosal surfaces. Thus intake of toxin at the epithelium also prevents the escape of toxin into the circulation and it being able to bind to potentially sensitive cells.

Additional evidence suggests that EHEC alters the environment in ways other than by immunosuppression. Neutrophils have been shown to have prolonged lifespan evoked by verotoxin-mediated inhibition of apoptosis (Liu et al., 1999; Liu et al., 1999). Persistence of

intimately attached pathogens is prolonged in animals with a lower rate of apoptosis in the epithelium (Magnuson et al., 2000) though the bacteria may not be causing the change in the proliferation rate but exploiting a change induced by another factor such as diet. The bacterial protein, CIF, a prophage encoded protein blocks the cell cycle of epithelial derived cell lines (Marches et al., 2003). This stops dephosphorylation of Cdk1 a driver of cellular mitosis (Nougayrede et al., 2005) and these cyclin dependent kinases combine with Cyclins which are integral to the cell cycle. These allow Cyclin/CDK complexes to enter the nucleus and phosphorylation of DNA binding protein complexes such as pRB/E2F occurs. Transcription of proliferation factors follows binding of these complexes to their DNA binding sites and induces proliferation of the cell. CDK1 in particular is important in the DNA synthesis when combined with Cyclin A (Johnson and Walker, 1999).

Other bacterial proteins may also be involved in this as CDT (Cytolethal distending factor) and CNF (Cell necrotising factor) (Nougayrede et al., 2005; Comayras et al., 1997; Caprioli et al., 1984; De Rycke J. et al., 1996; De Rycke J. et al., 1997) all have shown the ability to inhibit the cell cycle. CDT is a 3 subunit toxin that is found in a wide variety of Gram negative bacteria including *Campylobacter* and *Helicobacter* species, its full function in all species have not been elucidated however apoptosis of immune cells and the inhibition of fibrosis have both been suggested (Ceelen et al., 2006), and this may preserve the crypt in its native avoiding the healing process.

CNF is a protein toxin of 110-115 kilodaltons that is produced by certain *E. coli* isolates that have come from humans and cattle. There are a number of specific pathological responses that have been attributed to the protein and include enlargement of the cells which is accompanied by the formation of actin stress fibres. Additionally there is chromatin fragmentation and nuclear swelling as part of the distinctive pathology associated with this

toxin. However CNF also produces a block in the cell cycle at the G2/M stage (De Rycke J. et al., 1997).

These bacterial proteins are part of a group of proteins that are termed the cyclomodulins and these are used by bacteria to affect the host cell proliferative rate and influence the environment that the bacteria are attempting to colonise. They may help by impairing the expansion of immune cells and reducing the immune response. There may be changes in the epithelial integrity which may allow the bacteria to penetrate the host. Alternatively a reduction in cell turnover in the epithelium may also reduce shedding of cells and pathogens (Nougayrede et al., 2005). The actual effect is likely to be different depending on the specific area that colonisation occurs within.

The proteins discussed above are possible determinants and mechanisms for EHEC and the closely related EPEC to manipulate the host physiology and enable the bacterium to extend the period of colonisation within the host and this may allow for a greater spread amongst the host population or to other susceptible populations including humans but these proteins are not expressed ubiquitously throughout the serotypes of EHEC and EPEC.

1.8 The response of the host to bacterial colonisation.

Bacterial colonisation of the intestinal tract is not purely a result of bacterial activity but is a result both bacterial and host factors. These host responses can be protective and reduce or control the numbers and types of bacteria in the intestine with the host responding either with cellular or antibody mediated immunity, non specific responses can also be utilised to control bacterial numbers.

The intestine is a hollow muscular tube with an external serosal layer, underneath this is a muscular layer that is involved in peristalsis. The muscle consists of smooth muscle fibres that allow contraction both around the intestine and along the length of the intestine. Closer to the lumen is the sub mucosa and this is covered by simple epithelium and the large intestine in particular is covered in columnar epithelium. These cells are covered in a mucus layer that is produced by a subset of the epithelial cells, the goblet cells. In the centre of the tube is the lumen of the intestine and here is where ingesta is retained as well as the bacterial flora of the intestine reside and there may be a large number of bacteria resident ($\approx 10^{12}$ CFU per gram of intestinal contents). These structures contain many mechanisms to control bacteria within the gut.

1.8.1 Epithelial control of bacteria in the intestine.

The normal intestine contains bacterial flora and these interact with the host however the host is not normally in a state of uncontrolled inflammation in response to their presence. The host utilises a set of mechanisms to control these resident bacteria and any pathogens that may invade the host.

1.8.2 Innate immunity

Innate immunity is a term that is used to encompass the non-specific mechanisms that are able to control the microbial population in the intestine. These mechanisms respond to all classes of pathogens indiscriminately, and some are constantly active, others are induced by pathogens.

1.8.3 Mucociliary clearance

The surface of most mucosal surfaces is covered in a thick layer of mucus. This is true of the gastrointestinal tract and the upper respiratory tract. In the respiratory tract this layer lubricates the surface; prevent excessive moisture loss and trap bacteria. The bacteria so trapped are impeded in the ability to reach the epithelium. Cilia are cellular projections that are able to move in coordinated purposeful manner with cilia in the upper respiratory tract pushing mucus with its bacterial load towards the external environment. The bacterium are then moved away from locations where the pathogen may be able to attach, replicate and cause pathological changes such as epithelial hyperplasia , goblet cell hyperplasia and mucus overproduction (Boyton and Openshaw, 2002).

The mucus is made up of glycoproteins attached to the epithelium or cleaved from the surface of the epithelium (Deplancke and Gaskins, 2001; Lievin-Le Moal et al., 2005) and these are produced mainly in goblet cells and provide potential binding sites for bacteria. Cleavage of these proteins and the movement of the cilia (The Mucociliary elevator) reduce the load on the mucosal surfaces. Mucus is thought to provide more sites for bacterial colonisation and this binding may keep bacteria away from the epithelium. Ciliary cells are able to over express mucin genes in response to LPS in vitro (Zen et al., 2002).

Human intestinal cells respond in a similar manner to Listeriolysin O (Lievin-Le Moal et al., 2005) other bacterial proteins are implicated in altering the nature of mucus reviewed in Deplancke B and Gaskins H.R. 2001. This may allow bacteria to be removed away from the epithelium as mucus is lost from the system. By providing a large number of sites for bacteria to attach the chances may increase for successful attachment and if bacteria can inhibit mucus cleavage then removal of the bacteria and mucus may be impaired.

The intestinal epithelium also has a covering with mucus but there is no ciliary component to the microvilli of the epithelial cells. The peristaltic functions of the intestinal physiology replace that of the active ciliary function. The coordinated muscular contractions push the bacterial load gradually towards the anus where it may be evacuated without causing harm to the host.

1.8.4 Complement

The liver produces a vast range of proteins that are aimed at microbial clearance. These are known as complement and these proteins are found in all tissues including mucosal surfaces (Andoh et al., 1998). Binding of one of these proteins C3b activates a cascade that results in bacterial lysis. C3b is a spontaneous breakdown product of C3, which is protected from further degradation by LPS. If C3b is degraded then no further activation of the complement cascade can occur, but LPS protection from degradation allows binding of other proteins in the cascade. Accumulation of these proteins can then occur on the bacterial wall resulting in pore formation by the membrane attack complex (Firth et al., 2005; Gasque, 2004).

1.8.5 Antibacterial Peptides

Complement is an important protein but it is not the only protein involved in the control of bacteria within the mucoid layer covering the whole of the length of the intestine. This layer contains a vast array of proteins, all with differing functions, for example surfactants and enzymes; however three families of proteins are specifically involved with microbial killing: the- alpha and beta defensins and the cathelecidins. Antimicrobial peptides are found across all phylogenies such as reptiles, birds and mammals. Representing an early evolutionary adaptation to defend against disease by pathogens these are all short chain peptides that are capable of inserting into membranes and disrupting essential ionic gradients in the microbe (Lehrer et al., 1989) with the disruption of the inner membrane appearing to be the bactericidal event. The difference between the α and β defensins is based upon differences in the disulphide linkages and structural features (Fellermann and Stange, 2001). α-defensins have so far only been found in the intestinal epithelial cells. β-defensins are found in more widespread locations. Cathelecidins are the third class of antimicrobial peptides which were first identified and may have a role in the control of intimately attaching bacteria such as *Citrobacter rodentium* EPEC and EHEC (Iimura et al., 2005).

Although their main function, the activity of microbial peptides is not limited to direct killing. They have been shown to have activity in attracting and potentiating the activity of cells involved in bacterial killing (Bowdish et al., 2006) further functions described include stimulating wound healing. Expression of these proteins may be constitutively or may be induced by bacterial products (Hancock and Scott, 2000) including flagella (Schlee et al., 2007; Ogushi et al., 2004), probiotic bacteria are also able to induce these proteins and this may allow more rapid bacterial pathogen clearance (Wehkamp et al., 2004) or have roles in improving intestinal barrier function.

1.8.6 Physiological environment

The environment of the intestinal lumen enclosed by the epithelium can influence the survival of microbes substantially. In the stomach the severe acidic contents mean that many organisms will not survive the passage. Bile acids and pancreatic enzymes and pH changes all contribute to an environment that is difficult for pathogenic bacteria to colonise.

1.8.7 Colonisation resistance

As has been noted there are actually very few pathogenic bacteria in the host animal. As a percentage of the total microbial load it is probably insignificant in the normal animal. Commensal organisms inhabit space and deny pathogenic organisms a place to establish and replicate (colonisation resistance or competitive exclusion) (Ruas-Madiedo et al., 2006; Gueimonde et al., 2007; Lee et al., 2003; La Ragione and Woodward, 2003).

The presence and activity of commensal bacteria may also promote the virulence of pathogenic bacteria. *Salmonella enterica* use CFTR protein to translocate to the submucosa in the stomach and availability of this protein allows greater virulence of *Salmonellae* species. The distribution of this protein is influenced by the commensal flora particularly bacteriodes species such as ***Bacteroides thetaiotaomicron*** and this may promote susceptibility to the disease (Lyczak, 2003).

1.8.8 Cytokine induction and pathogen sensing.

Central to the activation of the specific measures in epithelial immune responses is the induction of a cytokine response. This allows the recruitment of immune cells to the appropriate location, not only does it switch on the immune system but it must also moderate, control and eventually switch off the response. Cytokine induction is initiated by binding of ligands to cell receptors. These ligands are commonly referred to as being proinflammatory and the most closely investigated receptors are the TLR receptors. There are 11 of these, each with differing ligands to which they can bind. Essential to this is the innate immune systems ability to recognise conserved regions amongst pathogens; these conserved regions are termed PAMPS (pathogen associated molecular patterns) (Akira, 2003; Akira et al., 2006), molecules that are common to a wide range of bacteria (figure 7) and allow the host to recognise a potential pathogen. Bacterial proinflammatory ligands that are bound include flagellin which binds TLR-5; lipopolysaccharide (LPS) which is bound by TLR-4; Peptidoglycan is bound by TLR-2, and TLR-9 binds bacterial DNA. Recognition is based on conserved motifs, not specific sequences as occurs in antibody or MHC molecules. Flagellin has been proposed as adhesin for EHEC and related bacteria therefore TLR-5 will be used as the example.

TLR receptor	Ligand
1	Diacyl lipopeptide
2	Triacyl lipopeptide
3	DsRNA
4	LPS
5	Flagellin
6	Triacyl lipopeptide
7	ssRNA/Imadazoquinolines
8	ssRNA/Imadazoquinolines
9	CpGDNA
10	Uropathogenic bacteria
11	unknown

Figure 7. Toll like receptors.

There are 11 TLR receptors identified and they have specific ligands (indicated above) to which they bind as part of their role in protecting against pathogens. The external domain consists of Leucine rich repeats with a TLR/interleukin 1 receptor (TIR) domain intracellularly. TLR-11 has an unknown ligand.

TLR-5 is a membrane bound protein that has been shown to be conserved in wide range of life forms (Takeda and Akira, 2004). These receptors have a cytoplasmic domain, and an extracellular domain. The cytoplasmic domain is similar to the intracellular domain of the Il-1 receptor family, while the extracellular portion contains leucine-rich repeats. As has been noted above, TLR-5 binds flagellin; this is an approximately 50 Kilodalton protein produced by bacteria and typically confers motility. However TLR-5 is found normally on the basolateral surface of normal epithelium. To interact one or other of these molecules must cross the epithelial layer. This may allow the host to remain unreactive to the flagellin of commensal bacteria. However, pathogenic bacteria will be more liable to cross the epithelium and allow TLR-5 to bind flagellin. TLR-5, while being the focus of much work, is not the only cell receptor that is capable of binding flagellin. ASGM, a

glycolipid on the cell surface has been shown to function as a receptor. It is possible that this binding activates cellular responses to induce the translocation or the production of TLR-5 on the apical surface. As discussed above in the pathogenesis of EHEC once bound the TLR receptors initiate a cascade of intracellular signals: MyD88 interacts with IRAK to ultimately activate NF-KB and induce expression of IL-8 and other pro-inflammatory mediators. Such a response occurs with various *E. coli* including EHEC (Rogers et al., 2003; Berin et al., 2002a; Zhou et al., 2003). IL-8 is thought to be the major chemokine involved in the recruitment of neutrophils to the sub epithelial space although MIP-2 has also been shown to have similar abilities (Ohtsuka et al., 2001) while other chemokines mediate crossing of the epithelium itself. The most common one is PEEC (pathogen elicited epithelial chemokine) or Hepoxilin-A3 (Mrsny et al., 2004; McCormick et al., 1998).

The normal assumption is that chemokines and cytokines are produced in response to active infection. The precise relationship between all the cytokines involved in the network defines the final response; however recent work on Il-8 levels in sheep suggests that there may be a constitutive secretion of Il-8 under specific circumstances. In normal sheep rectal epithelium there is a consistently high expression of Il-8, while the mucosa appears grossly and histologically normal (Sedgmen et al., 2002). It is therefore tempting to speculate that there is also an anti-inflammatory factor being expressed: for example Lipoxin analogs and presumably prostaglandins are able to fulfil this role (Gewirtz et al., 1998), similarly TGF-β is also able to inhibit lymphocyte activity (Ebert, 1999). Together such factors may lead to a physiological but not pathological inflammation. The full explanation of epithelial cytokine expression in the intestinal epithelium is still to be evaluated.

1.8.9 Phagocytic cells

Phagocytic cells include macrophages neutrophils and Dendritic cells. These all have the ability to ingest then digest microbes that they come into contact with. Cells with this ability can detect complement, the Fc component of antibody or to sample microbes directly. Once internalised, digestion of the microbe can occur, this can result in the control of the infection, however in some cell lineages but not neutrophils the degradation products can be associated with MHC class II molecules and are presented to the specific immune system. These cells are referred to as Antigen Presenting cells (APCs), and in the context of the MHC class II molecules, can present antigen to T-cells and B-cells. This leads to the induction and modulation of a specific immune response. Dendritic cells are important antigen presenting cells in the epithelium and present antigens to other cells involved in the immune system (Granucci and Ricciardi-Castagnoli, 2003). Once again this whole network of cells relies on the sending of complex messages via a cytokine network and other signals, for instance CCL20 is one of the chemokines that will mediate the attraction of Dendritic cells in response to flagellin binding (Sierro et al., 2001).

M-cells, while not phagocytic cells in the classically understood sense, are able to internalise bacteria and other antigens. The membranes of these cells are adapted to allow the adherence of antigens and enhance uptake of the antigen (Neutra, 1998; Niedergang and Kraehenbuhl, 2000). These cells translocate antigens and bacteria across the epithelium and deliver the bacteria to the T-cells and effector cell of the immune response.

1.8.9.1 Induction of apoptosis

Apoptosis is a natural mechanism for the removal of host cells that are no longer required and Phagocytic cells are involved in removing these dead cells. Apoptosis is a highly protective immune response and the induction of apoptosis is a feature of many bacterial infections, this has been investigated in *Salmonella* and *Legionella* species. In both of these it was possible to create mutants that did not induce apoptosis of the host cell and loss of this ability in *Salmonella typhimurium* (Monack et al., 1996) decreases the ability of the bacterium to disseminate through the epithelium. The apoptosis of epithelial cells allows control of bacterial infection by removal of infected or colonised cells. Intracellular bacteria have a particular requirement to avoid apoptosis and have developed mechanisms to avoid this, for example *Chlamydia pneumoniae* have been shown to inhibit apoptosis and this requires metabolically active reticulate bodies (Rajalingam et al., 2001). Other species such as *C trachomatis, C psittaci, and R. rickettsia* have been noted to inhibit apoptosis as well. Inhibition of apoptosis may be important to extracellular bacteria, for instance *EHEC* species are known to persist longer in animals that have been shown to have reduced apoptotic rates in intestinal epithelium; however a direct role for bacteria was not shown (Magnuson et al., 2000; Heczko et al., 2001) and it is unclear whether they actively modulate this rate. The bacterium *C. rodentium,* which is used as a model for EPEC and EHEC, has also been observed to behave in a similar manner (Vallance et al., 2003). Both EPEC and some EHEC produce CIF which can stop the cell cycle (Marches et al., 2003). Whether by protecting the cellular niche or reducing the loss of cells to which the bacteria binds it is clear that by altering the life span of the host cell a potentially valuable bacterial survival strategy is in place.

1.9 Monitoring of the host response.

EHEC and EPEC are thought to manipulate host responses to colonisation in a number of ways including immune modulation and suppression, induction of apoptosis and alteration of the host physiology. This can be achieved by the expression of bacterial proteins with the ability to affect pathways used by the host to control physiological functions by bacterial perturbation of gene expression. To monitor changes in gene expression within the cell a range of assays have been introduced.

The most commonly used assay is the Reverse transcriptase Polymerase chain reaction. It uses an enzyme to transcribe a DNA copy from RNA transcripts. Further amplification from this DNA copy can then occur to allow detectable levels of the transcript. These have been conventionally visualised by the addition of Ethidium bromide, which binds to the minor groove of the DNA molecule, to an agarose gel and allowing the products of the reactions to be exposed to Ultraviolet (UV) radiation. Due to toxicity problems these dyes have been gradually changing to other less toxic variants. This technique detects transcripts that are expressed within the cell even at extremely low levels within the cell. The unique nature of the DNA molecule allows for its replication and the increase in levels to above the detection threshold of many chemistries and devices. However quantification of the original level of transcript can be problematic as the plateau phase is reached in the commonly performed RT-PCR assays by 35-40 cycles. In an attempt to address this real time quantitative methods which incorporate the measurement of transcript levels within the PCR component of the assay and allow a measurement of relative or actual transcript levels. Two main chemistries are recognised at the current time: Dye-Quencher combinations and SYBR green chemistry. Dye quencher combinations are a

standard primer component and a probe component which has two molecules bonded to it - a fluorescent dye and a quencher molecule that keeps the dye quiescent. This pair is cleaved as part of the replication process with the fluorescence that results being measured. A curve of fluorescence values can be generated as cycles progress with greater fluorescence being seen as more transcripts are produced. SYBR green is similar to Ethidium bromide as it is a minor groove binding molecule. Stimulating the dye with lasers excites the dye to produce fluorescence when bound to double-stranded DNA, the increase in fluorescence is correlated to the level of expression of these genes. With both these chemistries the measurement that is utilised to give quantification is a threshold value. This is a level of fluorescence that lies within the log phase of the fluorescence values generated. By comparison with a known reference standard, quantities can be derived. Due to the sensitive nature of this technology it is critical that the source of the material is carefully considered and that contamination is eliminated.

In a single cell there may be thousands to millions of pathways that are active, some as part of normal host physiology and some as part of the experimental system. Some pathways may be completely abrogated while others may be only marginally influenced and there may be only slight changes within the pathways rather than on/off alterations. In a system where little is known it would take years to assay all known pathways using conventional assays. Microarray has been utilised to sidestep this limitation. Using the ability of DNA and RNA to replicate and bind with a high degree of fidelity whole sample isolates of RNA can be copied into DNA then assayed at a single time. The use of robotics has allowed thousands of spots of single stranded DNA to be bonded to a microscope slide each of those spots containing a unique sequence that is complementary to the specific sequence of a RNA transcript expressed by the experimental system. The RNA gathered from the

experiment is transcribed to DNA both to allow for greater stability and to allow the incorporation of a dye within the molecule. Binding of the DNA molecule produced to the appropriate spot allows detection by fluorescence based chemistries of the presence of that transcript within the experimental system. In addition comparison with the RNA derived from control animals allows the relative abundance of the transcripts to be calculated. In this way both upregulated and down regulated genes as well as those that are completely inhibited are able to be identified.

The material from which RNA is collected can be very influential in the outcome of RT-PCR, real-time and microarray experiment especially where low abundance transcripts are involved. Differing cells may respond to a common stimulus in a different manner, this may involve the same gene but in opposite directions and this may obscure a difference and if one cell type has a much larger population it may obscure the effect on the smaller population of cells. To avoid this many investigators have used an in vitro approach. This also potentially allows low abundance transcripts to be present in a significant number without amplification however this is extremely reductive and fails to take into account the influence of a plethora of cell types that interact within the host system and its environment that may not be reproducible in a lab based setting. Even Co-culture technique (growing mixed populations of cells) would only partially address this problem. Isolation of individual cell populations from a mixed and preferably in vivo situation is required to assay the cells within the host and ascertain the transcript profile of the cells of interest. Cells with a low population in the host for example Eosinophils may have a very different transcript profile from surrounding lymphocytes, an upregulation of any Eosinophil specific chemokines would be diluted and lost if all white blood cells were assayed, conversely a pure culture of Eosinophils may respond in a very different manner to a cell within the bloodstream. For

this example flow cytometry lends itself very well as a solution to this problem but for solid organs such as liver and intestine it is less straightforward. Homogenisation and rendering of the cells into single cell groups is required and the treatment to achieve this may in itself change the transcript profile, while the time delay to allow any such process will mean that transcripts of RNA will themselves decay and once again transcripts that are expressed at a low level will have their signal degraded rapidly in comparison to others. This approach has been used to investigate a number of bacterial host interactions (Kitadai et al., 2003; Hedegaard et al., 2007; Zhao et al., 2006; Ohno et al., 2006) including *Helicobacter, Salmonellae* and *Porphyromonas* species.

To address this problem has required a new approach and this has been Laser capture microdissection which has been utilised in many in vivo and IVOC experiments in recent years. This approach allows the identification of different cell populations and individual cells under a microscope and the removal of the cells onto a cap for downstream processing. This is achieved by the use of a near infra red laser of which is directed at a UPVC layer placed on the bottom of a transparent cap mounted in the light path of the microscope. As the light hits the plastic covering it melts the plastic which flows onto the cell below, adherence occurs and once the cap is lifted the cell detaches along the cell membrane as a complete unit without loss of cellular contents. As before care must be taken in preparation of the tissue to avoid degradation of the transcripts, however preparation of histological sections, which is the most common method of preparation, is more amenable to controlling these effects. This approach allows the identification of specific cell populations by recognition of the microscopic structures which characterise it, or by the use of immunohistochemical markers to identify specific cells. It also allows single cells to be assayed from complex tissues if downstream methodologies have the requisite sensitivity.

This approach has also been used to investigate host\pathogen interactions, species studied include *Helicobacter* in humans and resident bacteria in pigs (Resnick et al., 2006; Chowdhury et al., 2007).

The combination of Laser capture microdissection with microarray is an ideal approach to investigating pathological effects especially in systems where there is little information regarding changes induce by the pathogen. This approach allows the global transcriptome of a single cell type to be investigated without the response of other cell types obscuring the response. In vitro experiments allow a similar single cell type response but there are more interactions occurring within the host than pathogen/host interactions. There is an extensive molecular talk between all the cells in the host and this cannot be modelled in cell based model systems. In vivo experimental model systems do have all the interactions and using techniques to isolate out the cell type of interest allows the global transcript to be interrogated although the RNA extracted may require amplification to allow sufficient material for the experiments.

Hypothesis and Aims

EHEC exists in a complex environment in the host and it has a subtle effect in the host animal. There is limited information about the response to the bacteria by the bovine epithelium as there no or little pathological response noted in the reservoir animals or in the super-shedders that are thought to sustain colonisation in the herd. Shigatoxin has also not been shown to have a pathological role in the host and its true role in the bovine host has not yet been definitively described. An appreciation of the response induce by colonisation of the bovine host by this human pathogen will be useful in targeting the pathways that may be used by the bacteria to increase or extend colonisation.

The hypothesis investigated here is that *E.*coli O157:H7 modulates the host response in the absence of overt pathology with the aim of extending the period of colonisation within the animal. The specific aim is to examine the question of the bovine response to colonisation by elucidating some of the genes that respond to the colonisation of the intestine by *E.coli* O157:H7.

1. EHEC possess several pro-inflammatory factors that have been identified in the context of human epithelium. A model bovine epithelium system will be used in an attempt to define which bacterial factors may be pro- inflammatory and may have a pathogenic effect on the bovine host.
2. The response in the host is likely to be highly complex and mediated by signals from a multitude of sources. Since it is restricted to the mucosal surface EHEC is likely to directly influence intestinal epithelial cells. Epithelial responses *in vivo* will be monitored by isolating these cells to determine responses induced by EHEC. A combination of LCMD and microarray techniques will be used.

3. Microarray will generate a large amount of data and expression levels of selected genes will be validated by the development and use of qt-PCR assays.
4. Further verification will be carried out by detection of a phenotypic marker(s) correlating to deduced function(s) of gene products identified by microarray and qRT-PCR.

Chapter 2

Materials and Methods

2.0 General comments

The following section covers the materials and methods used in the thesis and is discussed as if done in an open Lab but Health and safety legislation requires that certain pathogens are handled at Biological containment III, which specifies the facilities required for working with these bacteria and the facility for conducting animal challenges; these conditions are defined by the competent authorities including local management and the Health and safety executive.

The sensitive nature of some of the techniques required clean room preparation and where necessary these were utilised; the preparation for microarray and real time techniques were both carried out in clean rooms. This avoided contamination with DNA, all reagents were stored in these clean rooms, targets and amplified PCR products were banned from these locations.

There is also significant legislation regarding animal welfare and ethical treatment of experimental animals. Ethical approval was gained from the Moredun Research Institute (MRI) ethics committee prior to the animal challenges. All staff involved in the experiments has the appropriate licences from the Home office and the experiment was licensed under the Moredun site licence. The welfare of the experimental animals was paramount and superseded all other considerations, prior to inclusion in the challenge all animals were examined by MRI Veterinary Surgeons, daily checks throughout the experiment were carried out by animal care assistants and the researcher conducted a clinical examination prior to conducting challenges and prior to post-mortem.

2.1 Bacterial strains:

The bacterial strains used in this thesis are outlined in the table below (Figure 8):-

Bacterial strain	Characteristics
Walla Walla 1	*E. coli* O157:H7 Verotoxin positive
Walla Walla 3	*E.coli* O157:H7 Verotoxin negative
NCTC12900	*E.coli* O157:H7 verotoxin negative (sourced from R. LaRagione VLA)
NCTC12900 FliC	*E.coli* O157:H7 Flagella negative. (sourced as above)
E. coli O5	With various H types
E. coli O111	Bovine derived strain
NCTC12900 EscN	EscN deletion in the TTSS. (sourced from A Roe)
105	Calf derived commensal *E. coli*
106	Calf derived commensal *E.coli*
Walla Walla 1 NalR	Naladixic acid resistant *E. coli* O157:H7 (S. Naylor)
Walla Walla 3 NalR	Naladixic acid resistant *E. coli* O157:H7 (Shiga toxin negative) (S. Naylor)
TUV933	*E. coli* O157:H7 strain (T Besser)

Figure 8.Strains used in the experiments described in this thesis

Important characteristics are briefly noted and discussed in more detail in the section concerned. These strains are sourced from various collaborators as noted.

2.2 Cell culture

To study the interaction of bacteria with the host requires a model system that simulates the natural infection as closely as possible. *E. coli* colonises the intestinal epithelium of cattle therefore a bovine derived intestinal epithelial cell line is the ideal challenge model; however no such line is available. A respiratory epithelium derived cell line exists; these embryonic bovine lung (EBL) cells were used as a cell model system to simulate the responses to bacteria and bacterial factors.

Cells were stored in a liquid nitrogen bank until required and revived using standard cell culture technique. The cells were grown in a medium consisting of minimal essential media eagle (MEM) (90% v/v) Foetal bovine Serum (10% v/v), and L-glutamine (1% v/v) and the cells were subcultured into 24 well plates. Once thawed the cells were diluted into growth media and centrifuged down at 1000g. A cell pellet was obtained and diluted in 10

ml, this mixture was aliquoted out as 0.5ml per well. Once the cells reached 100% confluency, this was followed by the replacement of the culture media with a starvation media consisting of MEM and L-glutamine alone (Serum free media) for 24 hours. The monolayers were then infected as outlined in the bacterial challenge procedure.

2.3 Bacterial challenge procedure.

Bacteria were removed from glycerol storage in a -70^{0}C freezer. The bacteria were plated out overnight. Where required the bacteria were grown in the presence of the appropriate antibiotic (indicated on the above table). A single colony was sub cultured into an overnight culture of MEM (hepes modified) (Sigma) in a shaking incubator at 200rpm. This overnight culture was diluted 1:10 and allowed to grow reaching an optical density of 0.3 to 0.4 in a shaking incubator at 200rpm. Optical density was measured by a Cecil 2041 spectrophotometer measuring absorbance at 600nm. To produce a multiplicity of infection of 100 this culture was again diluted 1:5 with MEM and 500µl of the final solution was used to challenge a 100% confluent EBL cell culture in a 24 well plate.

2.4.1 RT-PCR and qtPCR assay

The output from the cell model system was detection of IL-8 transcripts following challenge by bacteria on EBL cells. Transcripts are produced in response to stimuli that may be produced within minutes in comparison to proteins which are produced over a longer period. These small amounts are able to be copied and the amounts amplified to allow reliable detection of the transcript and multiple assays. The transcript can be visualised by a number of techniques. The following sections outline the process for producing readout from the challenged cells using RT-PCR or qtPCR.

2.4.2 RNA extraction

Extraction of mRNA was carried out following lysis of the cell monolayer with Qiagen lysis buffer and β-mercaptoethanol (10%v/v). The lysate was homogenized by the use of a Qiagen homogeniser. The total mixture was applied to the homogeniser column and spun for 2 minutes at 14000 rpm. Total RNA extraction was carried out using the RNeasy minikit (Qiagen) following the protocol supplied. RNA amounts extracted were determined on a Cecil 2041 spectrophotometer measuring absorbance at 280 and 260. tRNA in each sample was determined by the optical density measured at 260nm (OD@260) multiplied by 40 to give a value for the concentration in μg/ml. The sample could be stored after this step at -70°C if required.

2.4.3 Reverse Transcription of RNA extracts.

The RNA produced from the extraction is unstable and liable to degrade due to the presence of RNAses. DNA has better storage properties and is routinely amplified and visualised in laboratories worldwide.

Reverse transcription is required to convert RNA to DNA for downstream analysis. This was carried out at 37°C for 60 minutes using Qiagen reagents and enzymes made up as shown in the following table (Figure 9):-

Reagent	Final concentration
RT buffer	1 x
H20	
Oligo DT	1 μM
Omniscript	8 units per reaction
RNA	xμl required to give 0.4μg of total RNA
Nucleotide mix	0.5mmol each dNTP

Figure 9. Reverse transcription reagents

Reagents mix required to allow reverse transcription and creation of cDNA from RNA extracted from EBL cells. This is taken from the Qiagen instruction manual for the reverse transcription kit.

2.4.4 PCR amplification of target genes.

PCR was carried out on a Thermohybaid PCR cycler. The PCR mixture is described in figures 10 and 13 for the quantitative RTPCR and the PCR cycling conditions are laid out in figures 11 and 14. The primers used in both the RT-PCR and qtPCR amplification are set out in figure 12. The Taq enzyme and reagents were from Promega.

Reagent	Concentration (v/v)
Buffer solution	10
$MgCl_2$	6
dNTP	10
primers	1
H_2O	61.6
Taq polymerase	0.4
cDNA	10

Figure 10.Promega reagents used for all PCR reactions.

These were made up in the volumes indicated per reaction required. For example to test 3 experimental conditions the volumes are multiplied by 4 to allow for pipetting errors and negative controls.

	Step	Temp	Time
	1	94	10 minutes
Denaturing	2	94	45s
Annealing	3	56	60s
Extension	4	72	60s
	5	72	5 minutes
		35 cycles	

Figure 11. Thermal cycling programme for detection of IL-8 transcripts

Steps 2-4 were repeated 35 times to allow detection of transcripts. This was carried out on a Thermohybaid cycler.

To visualise the products of the PCR reaction an agarose gel was utilised, this was produced by mixing 1.1g of agarose with 100ml of Tris-borate EDTA (TBE) buffer and 1.1µl of ethiduim bromide. The mixture is heated in a microwave for 2 x 1 minute periods or until boiling, the molten mixture is then cast in a gel tray and a comb is used to create wells into which sample can be loaded. Once the gel has set sample can be loaded in the wells, a DNA ladder is also loaded to allow determination of product sizes. A voltage (100v) is applied across the gel for 45 minutes which ensures separation of the products and simple visualisation using a UV light.

Gene name / direction	Primer sequence
Interleukin 8 forward	TTCACAGCACTCGGAATCCT
Interleukin 8 reverse	ATGACTTCCAAACTGGCTGTT
Stx forward	TTTGATTGTTACAGTCAT
Stx reverse	GAAGGAAATAATTTATATGT
Angiopoetin-1 like protein forward	CCA TGA AGC AGT GAG TGC TGT C
Angiopoetin-1 like protein reverse	GTT CGG AAG ACC CAA AAT TCC
Jumonji domain containing protein 2B forward	ACG GAT CCT ACA GCG ACA ACC T
Jumonji domain containing protein 2B reverse	TCC TCG AAC TCC ACC TGG TAG A
Zinc finger 161 forward	CTG GTT AAG AGC GTC CCC TTT
Zinc finger 161 reverse	GCA AAA TGC AGG CCA AGA A
Cyclin C forward	TGC AAT GAA AGT GGA CAG AG
Cyclin C reverse	CAT TCC TAT CTT ACA GGT GTG C
Est-1p like protein forward	TCC CCA AAG CCC AAG ACT ATG
Est-1p like protein reverse	GAC CCT ATA TCC CAA AGC CCA
Actin forward	ACC AAC TGG GAC GAC ATG GA
Actin reverse	GAG CTT CTC CTT GAT GTC AC

Figure 12 Primer sequences.

These are for both qtRTPCR and RTPCR techniques used throughout the experiments.

Reagent	Volume	Final concentration
Mastermix	25µl	1x
Primer	Variable	0.3µM/for each primer
H2O	Variable	To give a final volume of 50µl
Template DNA	Variable	≤500ng/ reaction

Figure 13. Quantitect Sybr green mastermix

The reaction was made up to the table above. Approx 0.5µl was required for primers and template DNA depended on the concentration of each target gene.

Step	Temp	Time
Activation	95	15min
Denaturing	94	15s
Annealing	55	30s
Extension/	72	30s

Figure 14.Real time Cycling conditions.

All primers were cycled with these conditions and data acquisition where fluorescence levels were measured occurred at the extension step.

2.4.5 Real-time PCR

Initial assessment of the RT-PCR images suggested loss of virulence factors was not sufficient to completely remove transcript production in response to challenge by bacteria that were unable to express these factors. This change in relative amounts suggested that quantification of these factors should be attempted as there may be variance within the expression of IL-8 transcripts in response to the factors.

To quantify changes in gene expression real time PCR was used as this technique is able to theoretically measure the transcript levels down to a single RNA molecule. Using Primer express software (Applied Biosystems) the required primers listed in table 5 were designed; they were designed for all targets of interest. To allow standardisation of the assay the default settings in the programme cycling were used in designing all primer pairs, these are outlined in Figures 13 and 14. The primers were checked to ensure that a size difference between DNA and RNA derived products would be achieved. This was checked by Alignment on Web based software (Multalign (Corpet, 1988)) followed by utilising the primers in the reaction for standards to ensure single product of the expected size was produced. For all of the targets a DNA derived product was thousands of base pairs in size whereas the RNA targets were 100 to 200 base pairs in length. Plasmids were sequenced by the Functional genomics unit at Moredun using M13 primers to amplify the sequence of interest. BLAST was used to compare the sequence from the plasmid with archived sequences and the EST set from which it was designed. Additionally a melting curve was generated at each run on the real time PCR machine which allowed the detection of primer/dimer artefact, DNA contamination and multiple product sizes. Any primers that were found to produce these problems were discarded

Each run was done with the amplified RNA from 6 animals from the challenged group and 6 from the control group with each assayed in triplicate in an attempt top achieve statistal validity.

In order to allow quantification of the target genes we required a standard for each to allow comparison. To create the standard for the amplification the primers designed were used in a standard RT-PCR reaction composed of the same reaction mixture as previously described. The cycling conditions were those required for real time applications. The PCR products produced were cloned into the plasmid PCR 2.01 (Sigma) using standard techniques (Sambrook and Russell, 2001) PCR 2.01 plasmid was linearised, using TOPO cloning system, and the PCR products were incubated with the linearised plasmid to produce the standard. This was checked by repeating the PCR to detect inserts within the plasmid.

To calculate the standard the concentration of DNA using a spectrophotometer (i.e the plasmid) is measured and the copy number calculated from the following formula:-

X g/μl DNA / [plasmid length in basepairs x 660]) x 6.022 x 1023 = Y molecules/μl

The standard is then diluted 1:10 for 5 dilutions with each dilution assayed in triplicate to produce a standard curve (all techniques discussed from Qiagen and Sigma product manuals).

2.5 Analysis and normalisation of Qt-PCR results.

QtPCR revealed a large variation in the transcript level produced in response to challenge with a bias to lower values in a biological system. This was not unexpected and applying a logarithmic transformation to the values corrected for this.

Normalisation of these values was carried out to the values derived from the qtPCR techniques, this allowed for differences in the responsiveness and number of cells to be accounted for. Within the in vivo experiments there were very low abundance targets that were of interest. Thus a relatively minor difference in the number of cells or their relative activity could lead to a difference that was misleading. So it was decided to use a target from the microarray that was unchanged and of a similar transcript level to the targets of interest. To remove this bias we derived a correction factor from the median value to normalise the data. For each of the three replicates per animal an average value was calculated and these values produced a median for the group as a whole. Therefore 2 medians were found, challenged and controlled, and as these were different in qtRTPCR analysis but not in the microarray it is likely that a value between the two would more accurately correct for biases in the experimental system including differences in transcription and number of cells originally captured. For each animal the average of the house keeper values was divided by the median between both groups. This gave a correction factor that was applied to all further genes examined; experimental values were divided by this number to complete normalisation.

Once normalisation was completed, the median value for each animal was calculated as it is less sensitive to outlying values. These median values were used to calculate group median values, interquartile ranges and p-values for differences detected between the medians of the two groups. This approach was designed and discussed in conjunction with Jill Sales of BIOSS.

2.6 Isolation of commensal bacteria.

Host epithelial cells are exposed to bacteria within the normal intestine and it was considered useful to have some examples of these bacteria to test in the model system. These were derived from the faeces of healthy cattle. However EHEC bacteria are also found in healthy animals. To avoid testing these faeces were plated out on CT-SMAC plates (Sigma) which are selective for *E.coli* species and allow fermentation of sorbitol. O157 strains in the UK are unable to ferment sorbitol although German strains are able to ferment sorbitol (CDC website). Bacterial colonies that were sorbitol negative were tested for the presence of shigatoxin genes by PCR. The primers were derived from (Bastian et al., 1998).

2.7 Cattle challenges

The cattle for the challenge were sourced from farms in the local area. These were all approximately 6 weeks old at the time of challenge. Each animal was faecally screened for bacteria that were sorbitol fermenting. Any positive colonies were screened by PCR for the presence of verotoxin genes. Any calves that were positive were removed from the study.

All animals were clinically examined for good physical health prior to inclusion in the study and the animals were housed as a single group while awaiting challenge to allow normalisation of intestinal flora and any potential diseases status. Each animal was randomly allocated to 2 groups of 6 animals which were challenged by shiga toxin positive EHEC, 2 groups of 6 animals which were challenged with shiga toxin negative EHEC and a single control group of 8 animals. Those challenged with Shiga toxin positive positive EHEC took no further part in the study.

Cattle were challenged orally with 18 hr cultures of bacteria (100ml) prepared as outlined below and delivered by stomach tube. To ensure all of the bacteria entered the gastrointestinal tract and none remained in the tube 1 litre of prewarmed PBS (37^{0}C) wash used to flush the tube. The technique for challenging calves is derived from Naylor et al (Naylor et al., 2003).

2.7.1 Bacterial preparation

Bacteria were removed from glycerol storage and plated out on nalaxdic acid agar plates. These were incubated in the standard manner (37°C in a static incubator overnight). One colony was picked and inoculated into 20ml of LB with Nalaxdic acid (100µl/20ml) (Sigma-Aldrich), this was incubated overnight in a shaking incubator (200 rpm, 37°C). This 20 ml culture was kept warm for transport to the calves and administered by stomach tube.

2.7.2 Faecal sampling.

Each animal was sampled daily for bacterial excretion. 1g of faeces was diluted into 10ml prewarmed PBS and serial dilutions were made. 100ul of the serial dilutions were plated out, incubated overnight and counts were made the next day. Each of the dilutions was plated out in triplicate giving a total of nine plates in all. Counts of bacteria grown on the plate were multiplied by 10 times the dilution factor to give an estimate on cfu per gramme of faeces. The average of the 9 cfu/g estimates was calculated to give a daily value; this is recorded in the attached electronic data CD.

2.7.3 Post mortem sampling.

Once colonisation was established the animals were euthanased (day 10) by intravenous injection of pentobarbitone (euthatal 200mg/ml). The rectum and surrounding tissues were removed and placed into a variety of preservation media and storage conditions. These are listed below.

1. RNAlater™ (Ambion)
2. Zinc salt fixative
3. Formalin
4. Para formaldehyde
5. Liquid nitrogen freezing (Brass plugs gave the submucosal surface a flat aspect allowing orientation.
6. A fresh sample(5x5mm)

With the exception of the fresh sample all the samples were then archived in a -80^{0}C freezer until required. The fresh sample was placed in 1 ml of prewarmed PBS and briefly vortexed, serial dilutions were made and calculations were made as for the faecal counts; both these datasets are part of the database in the CD attached.

2.8 Laser Capture methods

2.8.1 Preparation of sections.

For each animal (6 in each group) a block of tissue was removed from the -80°C freezer. The brass plugs used at post mortem allowed simple orientation of the samples and cutting of sections in cross section. 5 sections were produced for each animal resulting in the production of 30 sections per group, a total of 60 sections were utilised for laser capture.

2.8.2 Staining of slides for Laser capture.

The slides were removed from the freezer and briefly allowed to thaw before being fixed, stained and dehydrated in accordance with the Arcturus manual. The protocol steps are listed in the below:-

1. Up to four slides were removed from the freezer and allowed to thaw for thirty seconds.
2. Seven jars were filled with the 25ml of the required solutions. From the HistoGene Frozen section staining kit (Arcturus).
3. The slides were placed in 75% ethanol supplied with the kit for 30 seconds, then into distilled water (also supplied) for 30 seconds.
4. 100µl of HistoGene staining solution was used to cover the section for 20 seconds.

5. Once stained the sections were again placed into distilled water for 30 seconds followed by transfer through gradually increasing concentrations of alcohol (up to 100%) for thirty seconds at each step.
6. The sections were transferred into Xylene for 5 minutes followed by air drying for 5 minutes.

Once stained the slides were placed in a box containing silica gel beads to prevent rehydration to be transported to the laser capture microscope.

2.8.3 Laser capture

The slides are placed on the microscope stage and visualised in the manner of a normal microscope. Once the section is deemed suitable and of sufficient quality the laser cap is placed in the line of sight. Pulse duration and strength is determined empirically for each section but 100mw was suitable in most cases.

A picture of each section prior to and after capture was obtained as was a picture of the material adherent to the cap. Examples of all of these are shown in the appropriate chapter of results and discussion. The caps were placed onto eppendorf tubes with 50 μl of RNAlater (Ambion) and placed onto ice prior to extraction. This was repeated for each of the 60 sections produced.

2.8.4 RNA extraction from captured material

Three methods were evaluated for RNA extraction

1. Trizol™ (sigma) extraction
2. Trizol™ extraction followed by use of Qiagen RNEASY minikit (as per Ark genomics website)
3. Picopure extraction (Arcturus)

1. Trizol protocol

Extracting RNA using Trizol was carried out by first washing the cap and adherent material 3 times in ice cold PBS to remove excess RNA later. 100µl of Trizol™ was added to the cap and the cap was incubated for 5 minutes at room temperature. 20µl of chloroform was added and the tube was shaken vigorously by hand for 15 seconds before incubating at 15 - 30°C (room temperature) for 2 - 3 minutes. Centrifuging the samples at 12000g (2 - 8°C) was used to separate the layers and this allowed the clear liquid at the top to be removed and placed in a fresh tube. 500µl of isopropyl alcohol was added to the clear solution and incubated in a fridge for 10 minutes The supernatant was removed and any pellet (where visible) was washed in 75% ethanol. The pellet was allowed to air dry before being resuspended in 100µl of RNase free water.

2. Dual extraction

Laser captured cells were initially extracted using the Trizol™ method described above. A Qiagen RNeasy kit was employed to carry out a further extraction as described above and as recommended by Ark genomics.

3. Picopure extraction.

The Picopure Kit (Arcturus) is a commercial kit specifically optimised to extract RNA from the cells captured by the laser capture microdissection technique. The process uses propriety buffers. The steps are outlined and follow the handbook provided with the Kit. However the cap was washed with PBS to remove the RNAlater that was applied to the caps and this is not a part of the standard protocol.

1. The cap was washed in PBS three times to remove residual RNAlater (Ambion).
2. Extraction buffer (50μL) was placed in a microcentrifuge tube and capped with the laser cap ensuring that the whole of the adherent material was covered.
3. Incubation was carried out at 42^{0}C for thirty minutes
4. The tubes were centrifuged at 800 x g for two minutes.
5. A preconditioned RNA extraction column was prepared by pipetting conditioning buffer onto the membrane in the column and incubating for five minutes at room temperature followed by a two minute centrifugation step at 16'000 x g.
6. 70% ethanol was mixed with the cell extract already prepared in a 1:1 ratio. (requiring 50μl)
7. The resulting mixture was placed on the RNA extraction column and spun at 100 x g for 2 minutes followed by 16'000 x g for 30 seconds.

8. A one minute wash with the supplied wash buffer (W1) was carried out (8000 x g).
9. 40µl of DNAse solution (Qiagen) was applied to the membrane and incubated for 15 minutes at room temperature.
10. A second wash with 40µl of wash buffer W1 was carried out.
11. Buffer W2 was used for the next wash at 8000 x g for one minute.
12. 11µl of elution buffer was applied to the membrane and incubated for one minute at room temperature. This was followed by one minute at 1000 x g centrifugation and immediately followed by 16000 x g.
13. The extracted RNA was stored at -80^{0}C until required

2.8.5 Assessment of RNA quantities extracted from laser captured cells.

Spectrophotometer assessment

The RNA was quantified by the use of a Cecil CE 2041 spectrophotometer. Absorbance was measured at 260 and 280nm with water or extraction buffer as a blank reference. 10µl of sample was measured in a cuvette and the optical density at 260nm was used to derive the concentration in the usual manner. RNA quantification of the amounts of RNA following extraction by the three methods gave the values in Figure 15.

Preparation	µg/ml
Trizol	0.328
Dual	0.164
Picopure	0.124

Figure 15.RNA extraction results from laser captured cells

Three protocols were followed and the results are presented. Although Trizol appeared to give more RNA the spectrophotometer results suggested that there was a great deal of contamination in this method.

Although the Trizol appeared to give more RNA by spectrophotometer it was decided that there was likely to be a lot of background staining in the hybridisation due to contaminants and a column extraction was the best way to eliminate this as recommended by Ark genomics. There was also felt to be the potential for contamination with the Trizol protocol on comparison to the Picopure method and as this has been used by other authors it was decided to use this commercial system.

2.8.6 Picochip analysis

Prior to amplification, labelling and hybridisation the RNA used in the microarray was measured and quality checked by a Picochip on an Agilent Bioanalyser. The method is as described in the manual. Samples were loaded into the wells and the RNA was separated along a gel/dye matrix. Fluorescence was measured by the Bioanalyser and the software was used to download the data into the files. Data is located in attached CD-ROMs.

2.8.7 Database and LIMS management.

There were large numbers of data sets and information to transcribe and cross reference for this analysis, to aid this and minimise transcription errors machine recording and machine readable formats were used. A Dell Axim pocket PC™ was used for data capture and files recorded on this device which allowed automated transfer to the access database.

The database was designed to capture all relevant to the analysis, this includes source of calves, sex and identification numbers. Bacterial colonisation data was also linked to the calf from which the faeces were collected by maintaining a reference number that was constant between all datasets (The primary key in Microsoft Access™). This allowed the creation of a database and is included in the Electronic data CD attached. It was also linked to the hybridisation slide and to the inventory of tissue stored in the lab for further experiments. This allows cross referencing of all information and opens the possibility of repeated iterations of analysis.

2.8.8 Preparation of cDNA and microarray analysis.

The amount of RNA captured from the cells of the epithelium is insufficient to allow microarray experiments to be performed. It is however possible to amplify the amount of RNA using a T7 promoter based system (Van Gelder et al., 1990) which increases the amount of material available for analysis. Several authors have recommended that this is carried out prior to real time assessment as it improves fidelity and sensitivity of microarray and qt-PCR assays (Park et al., 2004; Polacek et al., 2003). As we were using laser capture microdissection to isolate a specific cell population Amplification was considered mandatory to achieve enough antisense RNA to allow gene expression studies to be performed. This

was carried out in a manner similar to authors using the same techniques (Matsuzaki et al., 2004; Mohr et al., 2004; Thelen et al., 2004). Amplification was carried out by Ark genomics technicians and comprised an initial preparation of cDNA strand in a similar manner to that already described for the PCR experiments. However the enzyme required heating at 42^{0}C for 2 hours. The cDNA was taken forward to produce second strand DNA following incubation with DNA polymerase for 2 hours at 16^{0}C. This cDNA was purified using a commercial filter that is used as per manufacturers' instructions.

The microarray used was a 21000 cDNA array derived from EST sets produced by the Ark Genomics and MARC institutes. The array was used as a dye swap experiment with the RNA derived from each calf being hybridised to 2 microarray chips giving a total of 12 arrays hybridised. Hybridisation was carried out at 55^{0}C for 3 hours, 50^{0}C for 3 hours followed by a further 12 hours at 45^{0}C. This was followed by 5 washes at 50^{0}C for the medium stringency buffer, 5 washes at 42^{0}C for the high stringency buffer and 5 washes at 42^{0}C for the post wash buffer. In each chip each spot was replicated in triplicate to give technical replicates. The fluorescence data was extracted using a Genepix scanner running Bluefuse™ software and this software was used to conduct post processing (removing smear artefacts etc and ensuring correct identification of spots). This data was able to be loaded directly into Genespring where normalisation was produced by LOESS global normalisation as part of the programmes initial calculations. This platform was also utilised to carry out two-way ANOVA on the spot intensities within the arrays. This final data was split into upregulated or down regulated for further analysis and validation as required.

The microarray hybridisation was also carried out by Ark genomics as was scanning to acquire the pixel intensity data that is attached in the CD file. This was passed over as Bluefuse™ data files which were loaded onto Genespring software for analysis. This

package allowed normalisation by global LOESS techniques and ANOVA analysis of the changes in pixel intensity in the microarray.

2.9 Proteomics

The laser capture technique was employed to provide proteins for MALDI-TOF analysis of proteins expressed by the intestinal epithelium of the bovine. Using laser captured cells from each of the calves following the same protocol cells were obtained for proteomic analysis. The cells were lysed in lysis Buffer (7 mol/L urea, 2 mol/L thiourea, 4% w/v CHAPS,1% w/v dithiothreitol). This was applied directly to the caps which were placed in boiling water for 5 minutes. The caps in eppendorf tubes were spun at ≈10,000g for 5 minutes and this deposited the extracted protein in the eppendorf , this protocol was adapted from various literature references (Baker et al., 2005; Chaurand et al., 2004; Kwapiszewska et al., 2004; Craven et al., 2002)

Proteins in buffers were separated in a protein gel in the standard manner. Agarose gels were prepared by mixing 3ml of 1.5M Tris- EDTA,4ml acrylamide (40%) 5ml of distilled water and 40µl APS and 4µ TEMED. This was cast in an electrophoresis tank; a stacking gel was cast over the top (3.75ml 0.5M Tris-EDTA 9.75ml water, 1.5ml acrylamide, 150µl APS and 20µl TEMED). The extract was placed in lanes and a voltage of 135v was placed across the gel and run until blue stain from the leammli buffer run out of the bottom of the gel. The gels were stained by Colloidal blue by covering with dilute stain until bands appeared (approximately 30 minutes). 4 bands from control animals and 4 bands from the challenged animals were submitted to the functional genomics unit at the Moredun research institute for proteomic analysis using mass spectrometry.

2.9.1 Immunohistochemistry

Immunohistochemistry was used to determine the presence of cell proliferation proteins within the epithelium of the intestine of cattle.

Tissue samples were fixed in Para formaldehyde for 24 hours before being wax embedded. Once embedded these tissues could be archived long term in a fridge at 2-8°C. When required the sections were cut on a cryotome, placed on slides and dried in an incubator at 37°C overnight. All slides for a particular antigen were prepared at the same time.

These samples were dewaxed by three changes in Xylene of 3 minutes and three changes in water of 3 minutes. To expose hidden antigens, antigen retrieval was carried out using a domestic microwave. The slides with the sections were submerged in citrate buffer (ph 6.3). The buffer was heated twice for 10 minutes at full power in an 850W microwave.

Blocking of the section was carried out using the blocking buffer provided in the Dab Cytomation staining kit. This was carried out for 10 minutes; the buffer was washed from the slides with PBS before the primary antibody was applied for 30 minutes or overnight as required (Figure 16). The sections were washed in PBS before the secondary antibody was applied for 30 minutes. The DAB substrate was applied for 10 minutes. Slides were then stained routinely with haematoxylin and eosin. The slides were then mounted routinely. The technique is adapted from the Abcam website.

Antibody	Incubation Temp	Incubation Time
PCNA	4	Overnight
Retinoblastoma protein	37	30 minutes
Phosphorylated retinoblastoma protein	37	30 minutes

Figure 16. Antibodies used in the production of the Immunohistochemistry images in the thesis. The temperature at which they were incubated with the sections and the time required to give a good image.

2.9.2 Enumeration of the cells positive for colour change

To allow quantitative analysis of the cells positive for the colour change associated with the presence of the appropriate antigens counts were made of the cells within the epithelium. 5 crypts were identified from each animal which appeared to be complete. This was defined for the purposes of this study as having a shoulder at the top and a defined base ending in contact with the submucosa and being of sufficient length to fill the majority of a 40x power field.

Manual counting of the cells within these crypts was carried out and recorded electronically in an attempt to minimise transcription errors. Crypts were divided into upper and lower portions by approximation of the midway point. Both positive and negative cells were counted and percentages were derived from these raw figures.

To visualise these figures it was decided to represent these as interquartile ranges and a two sided student T-test was employed to evaluate the difference between medians of percentage values.

Chapter 3.

IL-8 transcription by bovine epithelial cells in vitro in response to challenge by *E. coli*

3.0 IL-8 transcription by bovine epithelial cell in vitro in response to challenge by *E. coli*

E. coli O157:H7 is the most well known of the Enterohemorraghic *E. coli* (EHEC) all of which are pathogens of humans. EHEC can cause a variety of clinical syndromes including diarrhoea, hemorrhagic colitis and haemolytic ureamic syndrome. These signs can be associated with a mortality rate which is normally less than 10% but can be much higher in individual outbreaks (Reilly, 1998). Several factors associated with the pathogenicity of EHEC have been identified as important in human disease. These include most prominently Shigatoxin (ST), LEE-encoded factors and flagella, though other factors may remain to be identified and may have as yet unrecognised roles. These aforementioned factors as well as numerous others have been extensively studied with respect to their effect on the human intestine and there are many tools which allow the study of their interaction with the intestinal epithelium. The intestinal epithelium is of importance in the pathology of bacterial/host and cell lines have been important in allowing the study of colonisation and initial interaction for enteric bacterial pathogens as it is a site of major pathology in enteric disease. In common with EPEC, EHEC causes tight junction disruption and neutrophil attraction at the site of colonisation; flagella has been identified as an important trigger for attraction of these neutrophils to the intestine, not only by EHEC but by *Salmonellae,* (Zhou et al., 2003; Berin et al., 2002a; Rogers et al., 2003) and many other bacteria. In addition to IL-8 other proteins such as CCL20 (Izadpanah et al., 2001) are involved in the attraction of polymorphonuclear leukocytes (Criss et al., 2001) . This attraction allows induction of a central part of the innate response and the production of a range of chemokines involved in the adaptive (or humoral) response.

E. coli O157:H7 associated disease is commonly reported as a result of contamination of the food chain by bovine faeces. Although numerous other routes of transmission are recorded, the origin is typically from faeces of reservoir hosts. In the national herd of the UK, approximately 8% of cattle and 24% of herds are carrying this bacterium with few if any animals showing clinical signs associated with this colonisation (Ogden et al., 2004). Therefore *E. coli* O157:H7 has been able to establish a niche within the bovine intestine amongst a plethora of other *E. coli* and other bacterial species resident in the gut. *E. coli* O157:H7 may thus be considered as a "commensal" bacterium however unlike most, if not all other commensal bacteria, *E. coli* O157:H7 is associated with histopathological lesions within the bovine intestinal tract. Specifically, these bacteria are able to induce the formation of attaching and effacing (A/E) lesions - as seen in the human - but there is no gross pathology or clinical disease associated with the lesions. This A/E lesion results from active rearrangement of the cell cytoskeleton initiated by bacterial proteins encoded in the LEE pathogenicity island alongside other bacterial proteins. This ability to induce change within the host cell cytoskeleton differentiates *E. coli* O157:H7 from many other commensal bacteria within the tract of the bovine; only *E. coli* bacterium and *Citrobacter* in mice have been reported in the literature as being able to cause these lesions, but many bacterial species including many serotypes of *E. coli* coexist with the host without causing pathological clinical signs. *E. coli* O157:H7 is therefore "commensal-like" with respect to the bovine host where it exists without causing overt clinical signs but unlike conventional commensals it causes a demonstrable change on the epithelial cell morphology.

To study whether bovine epithelium recognises *E. coli* O157:H7 as a pathogen and characterise factors that are important in the recognition of bacteria in the bovine gut an epithelial model system was employed. There is a paucity of cell lines available for studying

the interaction of bacteria and bovine epithelium. Therefore the use of an in vitro model is limited to the bovine derived cell line, Embryonic Bovine Lung epithelial cells (EBL). This cell line was utilised to examine IL-8 expression (as a mediator of the inflammatory response) of the bovine epithelial cells to *E. coli* O157 in comparison to EPEC and *E. coli* isolated from bovine faeces. The cellular polarisation and position of TLR receptors will influence the response to pathogens by the model however there is no published data regarding the position of the TLR receptors and the lack of knowledge of this information affects interpretation of data from these experiments.

The contribution of defined factors of O157 was assessed by the use of strains that were mutated in specific genes. This allowed identification of factors that are able to induce an IL-8 response as monitored by increased transcription. Induction of this chemokine is considered to correlate with inflammation as it is a chemoattractant molecule inducing neutrophil migration in response to pathogens.

The pro inflammatory effect of virulence factors expressed by EHEC in the bovine has yet to be fully determined. In this study it was possible to show that components of the bacteria are in fact able to induce inflammatory responses within the epithelium. These pro-inflammatory responses appear to require multiple factors of the bacterium. In contrast to human epithelium the appearance of attaching and effacing lesions is not associated with diarrhoea or other clinical signs. The presence of Shigatoxin in EHEC does not appear to affect the appearance of clinical signs in the bovine reservoir host.

3.1 Results

3.1.1 Induction of IL-8 mRNA by EHEC and EPEC

EHEC and EPEC strains were assessed for their ability to induce the transcription of IL-8 mRNA. The strains were all derived from outbreaks of EPEC disease, and Shigatoxin negative EHEC strains were also derived from natural outbreaks. EBL cells were challenged for three hours at a multiplicity of infection of 100 and at a temperature of 37 ^{0}C (5% CO_2) and assayed for Il-8 production by RT-PCR of the mRNA extracted from the epithelial cells. The resulting product was approximately 600bp in length and was separated on a 1.1% agarose gel. The gel (Fig.17) demonstrated a single product of the desired size. The amplicons in common with all the amplicons produced in this study was designed to cross intron-exon boundaries and would be expected to produce a very much larger product if DNA contamination was too be found. If DNA contamination was to occur then the production of Il-8 would be wrongly inferred, it would also cause difficulty in assessing the relative ability of various strains to induce Il-8 as differences between strains may be masked by transcription of DNA. It is believed however that the amplicons produced by the primers used in this study will produce larger amplicons than that of mRNA derived amplicons. All strains tested were able to induce transcription of IL-8.

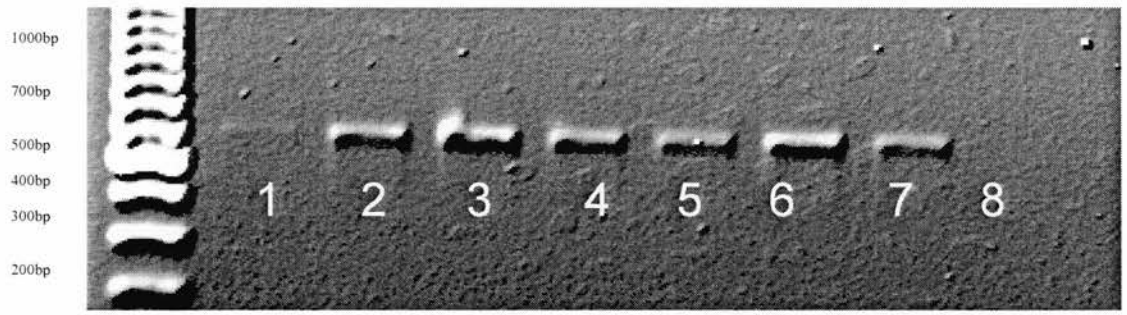


Figure 17. Il-8 mRNA transcription by EBL cells in response to challenge with a variety of E.coli serotypes.

EBl cells were unchallenged (lane 1); or challenged with VT+ve EHEC Walla Walla 1 (lane 2), Vt-ve EHEC Walla Walla 3 (Lane 3) ,E. coli O5: H- EHEC (Lane 4),. E. coli O5: Hun EHEC (Lane 5), E. coli O5 (lane 6), E. coli O111: NM (Lane 7), No template control (Lane 8). EBL cells were infected with the strains and mRNA harvested IL-8 levels were assayed. The levels of IL-8 between the groups were not visibly different between the various serotypes. This is a representative image from 3 experiments.

3.1.2 Assessment of the contribution of Shigatoxin to IL-8 induction.

Shigatoxin is a potent toxin that mediates inhibition of protein synthesis in cells that express Gb3 on their cell surface, Gb3 is a cell surface protein that allows internalisation of Stx and binding to ribosomes, cleavage of proteins means that Stx is likely to be the most important virulence factor in the human disease process. Its ability to cause inflammation in the bovine epithelium was here tested using a Stx positive strain (Walla Walla 1) and the isogenic Stx negative derivative (Walla Walla 3) which were used to challenge the EBL epithelial cells once again for 3 hours at a multiplicity of infection of 100. Figure 18 shows that there was no difference evident between the strains ability to induce Il-8 transcription when assayed by standard RT-PCR using the same protocol as described in the previous section.

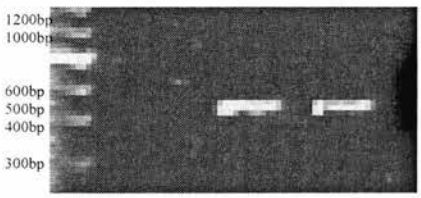


Figure 18. Il-8 mRNA transcription by EBL cells in response to challenge by E.coli with Shigatoxin. EBL cells were challenged as outlined in the material and methods with a VT negative (Walla Walla 3; Lane 3) and a VT positive isogenic strain of *E.coli* O157:H7. (Walla Walla 1; Lane 2). The product was separated on the gel and band intensity assessed by visual inspection. No difference in transcript levels was noted. Lane 1 represents a control (unchallenged cells, in which there is a slight expression of Il-8 transcripts. This is a representative image of 3 experiments.

3.1.3. Efa1 and ToxB contribution to Il-8 transcription by EBL cells

Efa1' and ToxB are two factors produced by EHEC (Tozzoli et al., 2005) that have undefined roles in colonisation within the host (Badea et al., 2003; Klapproth et al., 2005; Stevens et al., 2002). To determine whether either made any contribution to IL-8 induction by *E. coli* O157:H7, EBL cells were challenged with EHEC strains that were unable to produce one or both of these toxins; the cells were challenged at a multiplicity of infection of 100 and incubated under the standard conditions for 3 hours. The transcription of IL-8 was measured using conventional RT-PCR techniques and visualised on an agarose gel (Fig 19) where four bands of similar intensity were noted suggesting that neither ToxB nor Efa1 were able to substantially influence the transcription of Il-8 following challenge by EHEC.

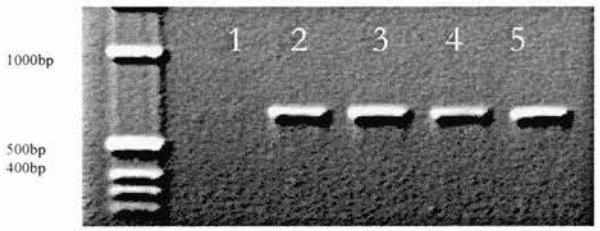


Figure 19. Il-8 response to ToxB and Efa1

Lane 1 Unchallenged cells; Lane 2 O157:H7; lane 3 ToxB deleted mutant; Lane 4 Efa1 deleted mutant; lane 5 isogenic variant lacking both Efa1 and ToxB. Strains that were unable to produce ToxB, or both appeared to produce equivalent Il-8 transcription in IL-8 cells to an isogenic wild type strain. This was able to increase transcription from control suggesting that factors other than ToxB or Efa1 were causing this transcription in EBL cells. This was a representative image of 3 experiments.

3.1.4 LEE encoded factors

LEE and associated factors have been associated with pathological responses. However there was no difference apparent in the gel images once compared to the actin reference (Figures 20 and 21). This suggests that in this study LEE is not a pro-inflammatory factor in cattle however in order to confirm this qtRTPCR was utilised. Figure 22 shows the variance in action controls that can occur and was used to inform decisions about whether differences in Il-8 transcription levels were real or artefactual and normalisation decisions.

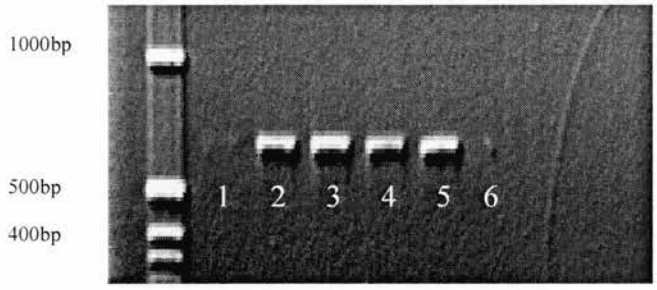


Figure 20.

Response to Bacteria with mutations in *escN*. These were therefore unable to translocate the LEE encoded factors due to a non functional ATPase component of the type three secretion system and were used to challenge EBL cells (Lanes 3, *E. coli* O157:H7 EscN-ve and 5 NCTC12900 EscN -ve). These were paired with their parent strains (Lanes 2, *E. coli* O157:H7 and 4 NCTC12900) no difference in the transcript levels could be determined by visual inspection. Lane 6 is a no template control. This a representative challenge of three experiments

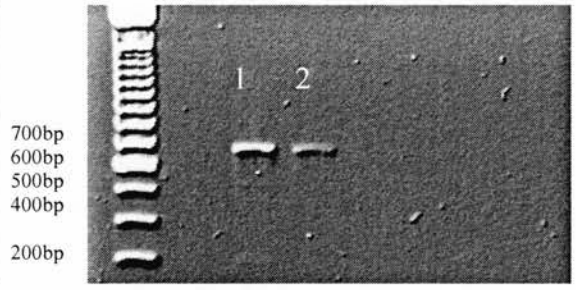


Figure 21.

Response to EDL933 LEE positive and negative strains. Lee positive EDL933 (Lane 2) and a derivative lacking the whole LEE region of the genome (Lane 3) were compared in the model system for their ability to induce IL-8 transcription. On visual inspection of this gel there is a difference in the two lanes but comparison of the actin gel for normalisation procedures suggested that there was no difference in EBL IL-8 transcription to challenge by either strain. This a representative challenge of three experiments

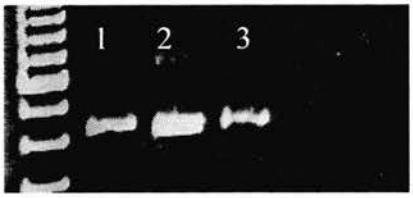


Figure 22. Actin controls

Actin control showing difference in levels suggesting differences in IL-8 transcript may be artefactual and informing decisions about normalisation. Lane 1 control Lane 2 wild type lane 3 EDL933 LEE negative.

The LEE Pathogenicity Island encodes a large number of proteins that have been implicated in the pathogenesis of human disease and in colonisation and persistence in bovine hosts (Dziva et al., 2004; Vlisidou et al., 2006b; Dziva et al., 2004; Vlisidou et al., 2006a). Since LEE components are thought to be important in pathogenesis it is possible that they may be involved in the induction of an inflammatory response by the bovine epithelium. LEE negative strains have been shown to have an increased level of proinflammatory responses in comparison to LEE positive strains (Rogers et al., 2003), however strains that were cured of the genes were no different in their ability to alter pro inflammatory responses when compared to the wild type. There may be other differences between LEE negative and LEE positive serotypes which account for the differences noted. Nevertheless there are a large number of proteins transported by the TTSS and these may have effects on the host, the exact expression of LEE encoded factors may define or fine-tune the response by the host. This may mean that there is only a minor difference in the chemokine response or other types of host response. There was no visible difference noted by the reverse transcription assay but as LEE has been shown to have an influence on epithelial cells by others, quantitative RT-PCR was undertaken to verify the results found.

The data from the quantitative RT-PCR were disappointing as they are from only one replicate; there was little difference between the strains suggesting that LEE has little effect in EBL cells (Figure 23) but no significance can be ascribed to this data.

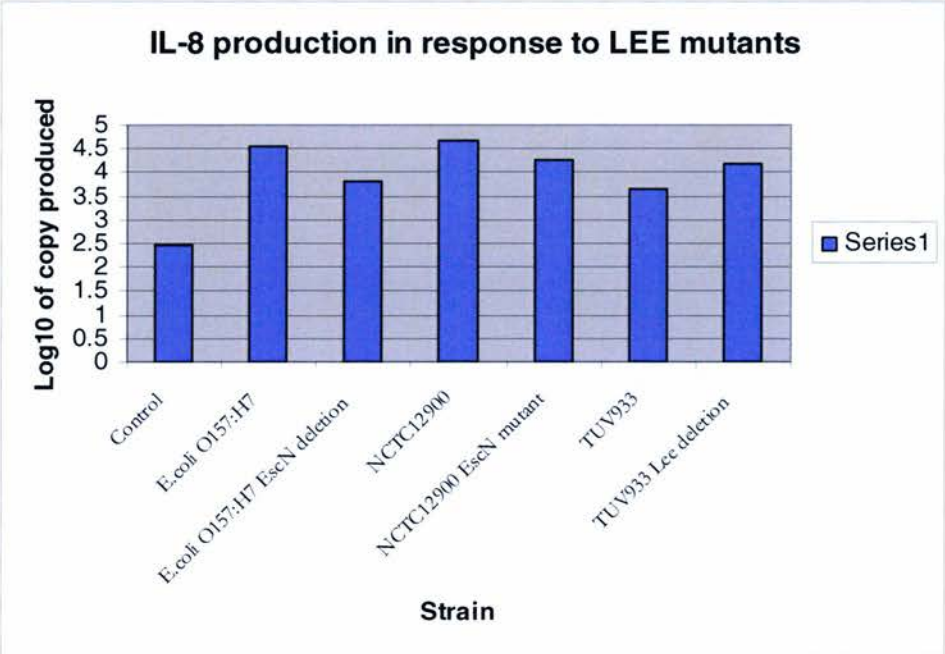


Figure 23. Response to LEE by Quantitative RT- PCR

The response by the epithelial model system to the wild type and the type three secretion system mutants of *E.coli*O157:H7 was tested. Two wild type strains *E, coli* O157:H7(Walla Walla 3) and NCTC12900 were compared to a strain with a non-functional component in the type three secretion system (EscN) This showed a slight reduction in the amount of IL-8.for each strain. Deletion of the entire LEE portion of the genome from TUV933 gave a slight increase in the IL-8 transcript production. The graph represents one experiment and due to the low number of replicates no inference can be drawn.

3.1.5 The contribution of H7 flagella to IL-8 induction by EHEC.

The flagella protein from *E. coli* is a major proinflammatory ligand for human epithelial cell systems (Melmed et al., 2003; Zhou et al., 2003). To test whether flagella similarly affect bovine epithelium model a flagella deficient mutant O157 strain and its parent strain were used. Both strains were supplied by R Laragione (VLA Weybridge). In the mutant strain the major flagellar subunit FliC was disrupted as described in the literature (Best et al., 2005). Challenges were carried out in the same manner as described above using the EBL model. As expected the wld type bacterium produced a response but the flagellar deficient strain produced slightly less IL-8 transcript. The magnitude of change was slight and further measurements were therefore made by QtRT-PCR to reinforce the phenotype.

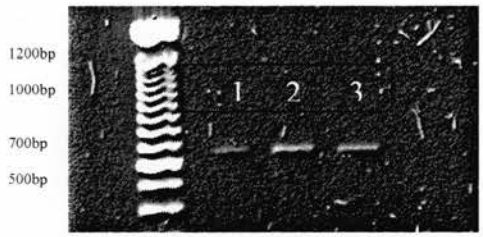


Figure 24. EBL responses to flagellate and aflagellate *E. coli* O157:H

Lane 1. Unchallenged, lane 2. challenged with *E. coli* O157:H7 NCTC12900, Lane 3 challenged with *E. coli* O157:H7 NCTC12900 *fliC* mutant, a mutant strain of EHEC deficient in the ability to produce Flagellin main subunit protein (*fliC*). EBL IL-8 transcript production in response to flagellate *E. coli* O157:H7 was increased in comparison *E. coli* lacking expression of this protein. This is a representative gel from three experiments.

RT-PCR is not quantitative therefore real time PCR was used to verify the expression of the IL-8 (Figure 25). This approach confirmed that IL-8 transcripts were significantly increased in both cells challenged with FliC negative strain and the parent strain ($p=0.0006$ and 0.0005 respectively) compared to unchallenged cells. The parent strain

NCTC12900 was able to induce the transcription of IL-8 to a significantly greater degree than the mutant strain *fliC* (p=0.001). Therefore flagella are likely to be potent inducers of inflammatory responses by bovine epithelial cells although they are not the only virulence factor involved in the induction of Il-8 as deletion of the flagellin gene does not completely abrogate the induction of Il-8 expression. Once again to primers for this assay were designed to cross intron exon boundaries in an effort to detect possible DNA contamination.

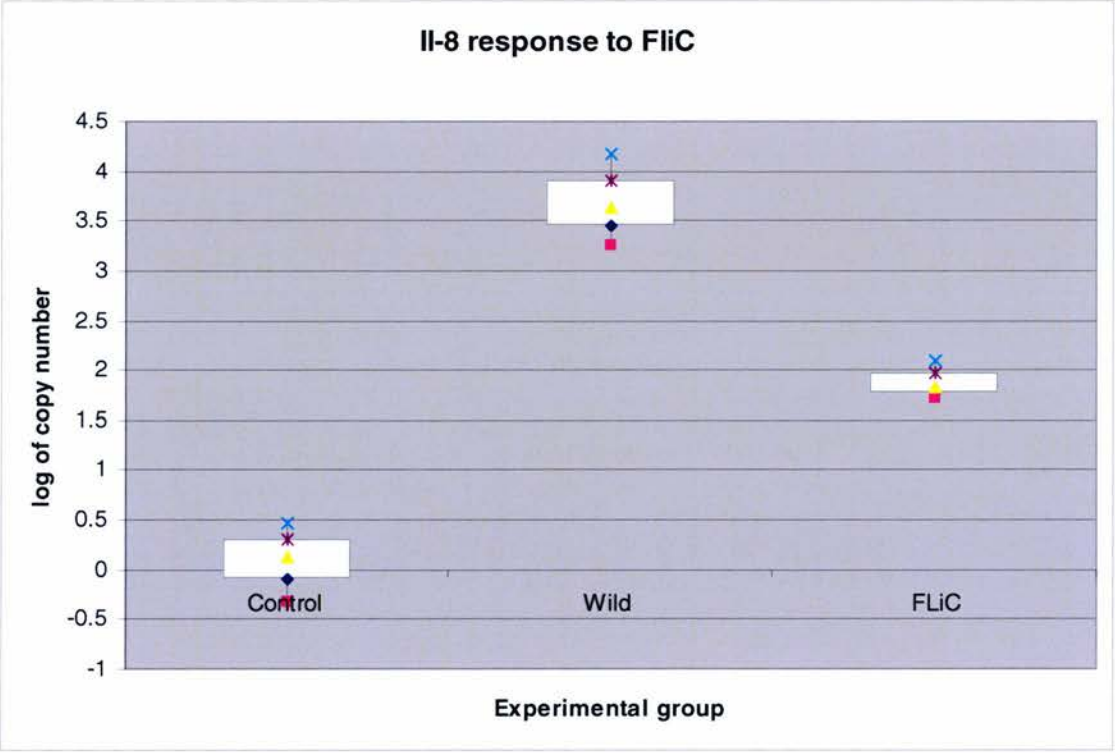


Figure 25. Il-8 response of EBL cells to colonisation by flagellate and aflagellate *E.coli* O157:H7

This allows quantative analysis s of the amount of transcript produced by the cells in response to challenge. A wild type was compared with both unchallenged and a flagellar deficient strain, fewer Il-8 transcripts were detected in the FliC strain. Though this abrogation was not able to return IL-8 responses to the same levels as control (unchallenged) cells, suggesting that there are other factors than flagella involved in the induction of Il-8 transcripts in this model system. This graph was produced from the results of three experiments

3.1.6 Comparison of IL-8 induction by *E. coli* O157:H7 and commensal *E. coli.*

E.coli including *E. coli* O157:H7 are normal residents in the cattle intestine and are not usually associated with gastrointestinal disease in this species. Therefore the capacity of resident *E. coli* to induce Il-8 was assessed, firstly faecal sampling was carried out on conventional calves and a large number of *E coli* were isolated (See Material and methods). As was found in previous challenges the *E. coli* O157:H7 strain was able to induce an increase in transcript levels for IL-8 ($p=0.006$), strain 105 was unable to induce Il-8 transcription above the level of control ($p=0.92$). There was rise in transcript production in EBL cells challenged with strain 106 but this was not shown to be significantly different from control ($p=0.49$) or from 105 ($p=0.49$) (Figure 26).

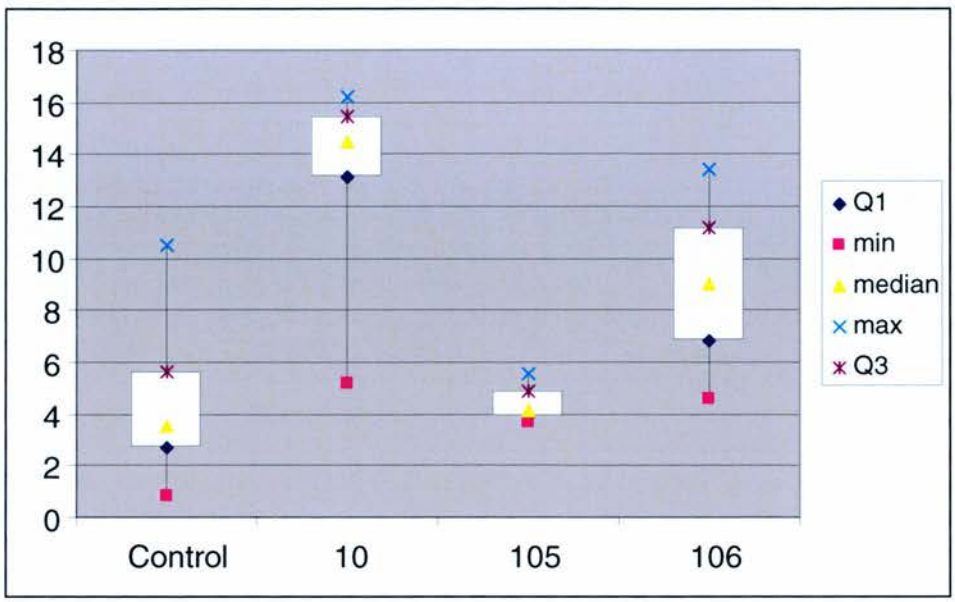


Figure 26. Qt-RT PCR analysis of IL-8 response to commensal colonisation.

Two *E.coli* isolates were obtained from the healthy conventionally reared calves. Faecal cultures were plated out on to selective agar and single colonies were tested for to eliminate O157 strains and VT carrying strains. These were used to infect EBL cells and qtRT-PCR was carried out to give log values of copies of mRNA produce by the cells. *E. coli* O157.H7 was able to elicit a significant number of copies (10) while 105, a faecal isolate was similar to control in the levels of IL-8 transcripts produced ($p=0.92$). 106 another faecal isolated was able to elicit less IL-8 transcript but this apparent downregulation did not reach statistical significance ($p=0.49$). Both of these bacteria were tested as motile by semi-solid agar and microscopy Each experiment was repeated three times to produce graph shown.

3.2 Discussion

Cattle colonised with *E. coli* O157:H7 are the usual reservoir for these bacteria and are grossly normal: unlike humans these animals show no clinical signs associated with colonisation by EHEC. This bacterium is found in a large number of herds and many individual cattle within the national herd and represents a risk to human health. Detection of EHEC in the cattle population requires more detailed investigation than other bacterial diseases that may be encountered in cattle such as *Salmonellae* which can cause symptoms in cattle as well as humans. In human disease there is a marked inflammatory response (Paton and Paton, 1998; Spears et al., 2006) with subsequent diarrhoea. Haemorrhagic colitis (HC) and haemolytic ureamic syndrome (HUS), the other recognised symptoms of EHEC-associated disease occur subsequent to this as Shigatoxin gains access to the circulation of the patient. In the human intestine *E. coli* O157:H7 is able to induce an inflammatory response following binding to the epithelium. Inflammation is a protective response to colonisation by pathogens and is not specific to EHEC disease; a variety of pathogens including *Salmonellae, Shigella* and viral pathogens induce a similar clinical picture of diarrhoea and this allows clearance of these pathogens from the intestine and usually resolution of the infection. Histologically there is an increase in the neutrophils in the infected tissue, however in cattle there is no evidence of this or of oedema or swelling of the site of colonisation, although virulence factors are expressed within the bovine host and there are histological changes associated with colonisation. Although some of these virulence factors have been associated with changes in the colonisation dynamics (R Laragione pers comm..) and there is evidence from mutagenesis studies that some of these factors, including EscN, NleD and fimbrial proteins, may be important in promoting colonisation (Dziva et

al., 2004), there is little evidence to demonstrate a potentially pro-inflammatory role in the bovine. Therefore using a bovine epithelial model system the ability of selected virulence factors to induce an inflammatory response was examined. The output for this model system was defined as IL-8 transcript production and was assessed by reverse transcription Polymerase chain reaction. The primers were designed to cross intron- exon boundaries and therefore by detecting the difference in base pair sizes it is possible to determine the presence of DNA contamination in the samples tested. There were no extraneous bands detected in any of the samples suggesting that DNA contamination was minimal and unlikely to obscure differences in perceived transcript production. Production of amplicons from DNA would be liable to give a false positive band on the gels or on the real time PCR data therefore Il-8 mRNA transcription would be inferred where none had in fact occurred. This may lead to difference being obscured or emphasise and would makeethe interpretion of data impossible. As stated the primers were designed to produce amplicons of differing sizes in the face of DNA contamination and avoid this complication of interpretation.

Like EHEC, EPEC are a cause of profuse watery diarrhoea in both humans and animals however as EPEC lack Shigatoxin therefore HC and HUS are not associated with this infection. Attaching and effacing lesions are common to both infections; EPEC and EHEC share similar virulence factors and the pathogenesis is thought to be similar though not identical. A panel of EPEC and EHEC were used to challenge EBL cells and the ability to induce transcription of IL-8, these included strains of a variety of serotypes that were able to elaborate Shigatoxin. This panel of bacteria were all able to induce transcription of IL-8 from the challenged EBL cells and there was no obvious difference between any of the strains. It has not previously been demonstrated in a bovine model that pro-inflammatory response to these pathotypes is similar. This induction of IL-8 suggests that there are pro

inflammatory virulence factors carried by the bacteria that are at least capable of inducing inflammation within the host. The results suggest that the major pro-inflammatory factors are common to both EPEC and EHEC as there is little difference observed in transcript levels, due to the similarity in virulence factors between the two types of bacteria.

The interaction of the bacteria with the epithelium causes inflammatory responses and histopathological changes in the epithelium. The pathological changes occur in response to the presence of virulence factors associated with pathogens. There are a large number of bacterial components that can be detected by epithelial cells as receptors for bacterial components exist, and these can detect a varied group of components including bacterial DNA, LPS, and flagella. These bacterial components are recognised by a family of host proteins that are expressed in almost all animal families (Takeda et al., 2003), called the Toll-like receptors (TLR) and the NOD family of proteins. These proteins initiate a series of cascades that result in the release of pro-inflammatory chemokines which initiate and propagate inflammatory responses and which can act as a marker for inflammatory responses. This inflammatory response leads to the attraction of phagocytic cells: the initiation of protective immune response, both adaptive and humoral, and the resolution of infection unless an overwhelming assault occurs. In addition to the components that are common to all *E. coli* bacteria including residents EHEC have specific virulence factors including Shigatoxin, LEE encoded proteins, haemolysin and Efa1' and ToxB some of which appear to have functions in the pathogenesis of human enteric disease. The contributions of these individual virulence factors is unclear in the bovine host and as these may be important allowing colonisation of the reservoir host as well as causing pathogenesis in the human these factors were tested in a model system to ascertain if they had a pro-inflammatory role in the bovine.

Shigatoxin is one of the defining virulence factors associated with *E. coli* O157:H7. It has profound effects on cells expressing its receptor, Gb3, for instance in the endothelium of human blood vessels contained in the gut and renal vasculature. However cells positive for Gb3 are present in the bovine intestinal epithelium (Hoey et al., 2002). In the endothelial cells in the human intestine and renal system protein elongation is blocked by the A subunit of the toxin (Lingwood, 1996). However differences in the intracellular domains of Gb3 in the cattle epithelium mean that the toxin does not reach the ribosome but is directed to endosomes/lysosomes. In ligated gut models of *E. coli* O157:H7 it was shown that in such as fluid accumulation and neutrophil attraction (Stevens et al., 2002) shigatoxin deficient strains were as efficient in producing these enteropathogenic responses as the wild type however there may still be a role for shigatoxin in the induction of innate immunity. The epithelium contains a subset of cells of lymphocyte lineage and these are thought to be influenced by the presence of shigatoxin. Shigatoxin has been shown to induce the production of chemokines (Thorpe et al., 1999) but may not be the most important of the virulence factors with respect to epithelial inflammation ((Berin et al., 2002b) (Miyamoto et al., 2006). However it may have differing effects dependant on the cell background as recent work suggests that while it induces endothelial cell death its effects on human enterocytes may be a more subtle decrease in the inflammatory response (Gobert et al., 2007). Stx may well have a similarly subtle effect on bovine enterocytes. Shigatoxin may also be involved in colonisation of the bovine intestinal epithelial cells and this may be its role in the bovine rather than inducing inflammatory responses (Robinson et al., 2006). Should shigatoxin be a major pro-inflammatory ligand within the epithelial model system there would be an increase in the amount of Il-8 transcripts detected. In fact the RT data suggests that there is no difference in the amount of transcript detectable. The function of shigatoxin in the model

system may not be to influence the production of IL-8, but may in fact be more important in colonisation or other processes. Gb3 has not been demonstrated in EBL cells so it is difficult to ascertain whether the similarity in response between the two strains is due to a lack of receptor or whether bovine cells are truly unresponsive to the toxin although lack of Gb3 receptors may not preclude responses as demonstrated by Gobert et al.

The LEE pathogenicity island encodes a number of virulence factors which are injected into the cytosol, these proteins alter the cytoskeleton of the cell and allow the formation of the attaching and effacing lesions. The LEE region of the genome, using bacteria deficient in either the whole Type Three Secretory System or with the energisation of that system rendered non-functional, was examined utilising standard RT-PCR techniques. The gel images gave bands of similar intensities suggesting that when either the whole TTSS was deleted from the genome or the TTSS was rendered unable to deliver the translocated proteins (Tir and the Esp family of proteins as well as around 30 other proteins) (Tobe et al., 2006)into the host cytosol this was not able to induce a change in IL-8 transcription in comparison to the wild type. There is evidence that LEE may have a role in modifying the response by the epithelium although there may be other differences between the strains tested in that study (Rogers et al., 2003). EspB proteins have been demonstrated to induce a suppression of pro inflammatory responses through down regulation of Nf-$\varkappa$B (Hauf and Chakraborty, 2003) though whether EspB is required for translocation of the effector protein or is able to produce this effect is unclear . The data from the qtRT-PCR presented here though not definitive is suggestive that a similar role is occurring in the bovine. The LEE pathogenicity island is essential in producing the attaching and effacing lesions associated with EHEC associated disease. The qtRT-PCR data does not have sufficient power as a study to allow assignment of significance however the down regulation

is suggestive in that there is agreement with the literature, more replicates would allow this to be confirmed in the bovine.

ToxB is encoded on the pO157 plasmid and has an uncertain role in the colonisation of the host by O157. LifA a virulence factor in EPEC has been implicated in the down-regulation of proliferation of bovine lymphocytes (Abu-Median et al., 2006) and homologues of these proteins have been demonstrated in both O157 strains (efa1') and in non O157 EHEC strains Efa1, however the truncated variant present in *E. coli* O157:H7 does not appear to have a similar role. It was hypothesised that ToxB or Efa1' might contribute to colonisation by influencing the inflammatory response. By challenging with strains that were deficient in either ToxB, Efa1' or both the contribution of these proteins was assessed. There was no difference noted on the gel obtained from this technique (Fig 19) suggesting that these factors did not have a substantial effect on IL-8 transcription by epithelial cells and by implication by the inflammatory response. However it is noted that the effects already noted in the literature are as a result of the action on lymphocytes, and these occur within the epithelium (as intraepithelial lymphocytes, IEC) and it may be on this specific cell subpopulation rather than epithelium that these virulence factors are acting upon in vivo. Lymphocytes that are challenged by potential pathogens are likely to proliferate and signal to the immune system, by reducing proliferation of this subset EHEC may protect itself from immune surveillance by IEC that are integrated into the epithelium.

Flagellin has been identified as a major proinflammatory ligand in the human response to colonisation by EHEC (Miyamoto et al., 2006; Berin et al., 2002a; Rogers et al., 2006). It has also been implicated in the pathogenesis of many other bacteria particularly *Salmonellae spp.* (Eaves-Pyles et al., 2001). It was hypothesised that this pro inflammatory activity might also be apparent in bovine EBL cells when challenged with *E. coli* O157:H7 in

comparison to a strain unable to express flagellin (FliC). Using standard RT-PCR methodology it was demonstrated that there was a difference in the IL-8 transcripts produced by the EBL cells when challenged with a flagellin-deficient *E. coli* O157 compared to the parent flagella producing *E. coli* O157:H7 strain. To confirm and quantify this change a real time assay for IL-8 was developed; using this system a reduction in the amount of IL-8 transcripts was produced when a FliC-deficient strain was used to challenge EBL cells in comparison to the wild type flagella positive strain. However there is still evidence of Il-8 transcript production from both the standard and the quantitative technique. Evidently, as in human epithelium, H7 flagella are major pro-inflammatory ligands for bovine epithelial cells therefore cattle do indeed recognise and respond to this signal. That said, it is evident from the induction of Il-8 by aflagellate *E.coli* O157 that there are other bacterial factors that may be proinflammatory. It is likely that these are some of the virulence factors already discussed but their contribution in flagellate bacterium may be obscured by the overwhelming production of Il-8 induced by flagellin. It is possible that challenge with "commensal" *E. coli* with the ability to express these factors may produce limited IL-8 transcripts and support this suggestion.

E.coli O157 is commensal-like in cattle and in common with many of the commensal *E.coli* it expresses flagella. The normal resident bacteria commonly referred to as commensals produce no clinically significant signs within the host and *E. coli* O157:H7shares this characteristic. Therefore bacteria from bovine faeces were isolated to be used as representative of the commensal *E. coli* flora of the bovine gut and were compared against Walla Walla 3, a non-shiga-toxigenic strain of *E. coli* O157:H7. EBL cells were challenged with one strain, 105 which displayed a non sorbitol-fermenting non-motile phenotype. When examined microscopically this strain was demonstrated to be non-motile (visual inspection

of warm growing cultures allowed determination of the ability to move). This phenotype, which suggests this strain lacks functional flagella although it is possible that the strain is lacking in some other component which is required to promote motility, produced no more transcript than unchallenged EBL cells suggesting that this resident *E. coli* is unable to induce IL-8 transcript production in the model system. Isolate 105 resembles the aflagellate O157 strain previously discussed in its inability to demonstrate motility, however whereas this mutant was able to induce some transcript production suggesting that it still has a pro-inflammatory role in contrast to 105 which appears to be have no pro-inflammatory activity. Strain 106 was motile when examined microscopically in contrast to 105 and there appeared to be an increase in production of Il-8 transcript when EBL cells were challenged with this strain of *E coli*. The change in transcript level was not significant either as compared to control ($p=0.49$) or when compared to *E.coli* O157:H7 ($p=0.26$). This suggests that flagella is not the sole component required to initiate IL-8 transcript production and that other virulence factors are required to induce inflammatory responses.

Flagellin induces IL-8 through interaction with Toll like receptor 5 (TLR-5), one of the toll like receptors. Toll like receptors are cell surface receptors that are able to bind components of bacteria and viruses and in doing so are able to initiate a protective response by the innate immune system (Akira et al., 2006). These pattern recognition receptors bind pathogen components through intracellular interactions focussing on the nuclear factor NF kappaB (NF-κB). This transcription factor induces the expression of chemokines principally Interleukin 8 (Il-8) which act as chemoattractants and induce neutrophil to the site of colonisation. This class of proteins is an important one in initial steps in the control of pathogens within the intestine by recognising their presence and initiating the responses that control the infection. These responses include the inducton of antibacterial peptides

(Thoma-Uszynski et al., 2001), the recruitment of phagocytic cells (Yu et al., 2004). Another family of pattern recognition receptors includes the NOD family, which act in a similar manner to the Toll family although these exist intracellularly and may allow recognition of those bacteria that are invasive and evade the Toll receptor family (Kim et al., 2004) by recognition of Peptidoglycan fragments (Viala et al., 2004).

There are a great deal of factors capable of producing an inflammatory response and these bacterial factors are usually referred to as virulence factors. Some of these factors are recognised by components of the innate immune system including Toll and NOD receptors and recognition of pathogens can therefore occur before the humoral immune system becomes activated. All of the Toll receptors have a specific distribution about the cell, Toll receptor 5 (TLR5) is distributed on the basolateral surface of the cells (Gewirtz et al., 2001) in intact epithelium and this should isolate it from flagella on the apical surface. However as the state of polarisation and the location of TLR5 is unknown in EBL cells at this time it is difficult to postulate a mechanism to explain the differences between the response to the wild type O157 strain and the response to commensals and flagellar deficient strains. Further investigation to define the position of the toll like receptor on this model system is required; additionally examination of the cell tight junctions to define whether polarisation of cell structures occurs, histological examination of tight junction proteins and TLR5 polarisation along with transepithelial resistance would allow validation of observations within this model system. This separation may allow tolerance towards commensals, as FliC has been demonstrated to increase transcription in challenged EBL cells it is possible that the LEE Pathogenicity Island has some role in promoting inflammation in the bovine. This component's role in disrupting epithelial cell junctions may allow flagella to cross the epithelium which by interacting with the receptor TLR5 may result in an increase in the IL-8

transcription demonstrated in this study. The role of flagella is thought to be as a result of Toll-5 interaction but in this model system could be a result of the flagella promoting interaction with cell surface by motility allowing other bacterial proteins to interact with the cell surface. Motility deficient mutants are available which could be used to test the motility component of the flagella. If the motility acquired by the presence of this structure is the significant component of the ability to induce inflammation it is likely that a strain lacking motility will induce a lower level of inflammation than the wild type. Centrifuging these strains to ensure contact with the epithelial model would reinstate the proinflammatory response seen by the wild type if motility is required to produce pathogenesis.

This data is in contrast to other studies that have been carried out utilising non-pathogenic (commensal or resident) bacteria in human and murine cell models systems for instance Bambou et al were not able to convincingly show up regulation of Il-8 or KC (a murine analogue) chemokines involved in the inflammatory response (Bambou et al., 2004). As a true commensal is found in the gut of the host, the strains used by other studies may not be representative of this type of bacteria. MG1655 is a lab adapted human isolate whereas the bacteria used in this study are derived from the bovine gut and have undergone a very limited number of passages (no more than 5 in total). They were used to colonise a model system based on the host from which the bacteria were originally derived so both bacteria and cells may show adaptation to each other. More critically in the study of Bambou et al. Toll- 5 was demonstrated as being apically present in the organ cultures however TLR5 has been demonstrated in intact normal epithelium TLR5 as being retained on the basolateral surfaces of intestinal epithelium by other studies(Gewirtz et al., 2001). TLR5 responsiveness is thought to require ancillary receptors and signals (Ogushi et al., 2004; Tallant et al., 2004) though the importance of co receptors is not yet finalised (West et al.,

2005). In the model described here there is little evidence for bacteria being able to elicit an inflammatory response as the qtRTPCR data does not support the hypothesis that commensal bacterium are able to induce IL-8 transcription. Recognition of the O157 strain, which carries multiple virulence factors, by EBL cells does occur and this is in contrast to *E. coli* O157:H7 which is able to induce IL-8 transcription. In the host there is no evidence of inflammatory response as many serotypes can be carried in healthy cattle (Blanco et al., 2005; Blanco et al., 1997).

Many *E. coli* can be carried in the gut asymptomatically and some may be beneficial for example Nissle 1917 is a strain of *E. coli* that was originally isolated as a resident in humans and has been used as a probiotic in humans and as a therapy for inflammatory bowel disease (Kruis et al., 2004), and in humans and mice has been shown to have an effect on the clinical presentation of inflammatory bowel disease and ulcerative colitis (Schultz et al., 2004; Rembacken et al., 1999). *In vitro* it has been demonstrated that Nissle is able to induce chemokines (Lammers et al., 2002; Helwig et al., 2006; Ukena et al., 2005) including Il-8, Il-10 and MCP-1. Additionally flagellin of Nissle 1917 is able to stimulate the production of beta defensin-2 , one of a group of pore forming proteins that insert into bacterial membranes and disrupt chemical gradients (Schlee et al., 2007; Wehkamp et al., 2004). This in part resembles EHEC as it too has the ability to up regulate some pro-inflammatory cytokines, principally Il-8, therefore Nissle and possibly other commensal bacteria may have components that are recognised by the host. In addition to an ability to influence chemokine production and possibly alter the host towards tolerance of bacterial colonisation, Nissle may have other effects on the epithelial cells in promoting and altering the tight junctions of epithelial cells (Zyrek et al., 2007a), in comparison to EHEC in the human which severely damages these structures, and produces repair of tight junctions. This

may restore cellular polarity and allow the IEC to maintain an asymmetric distribution of cell surface proteins. Similarly the resident strains examined here may have no or little effect in the inflammatory process but function in maintaining the tight junctions in the face of disruption by EHEC and EPEC strains, thus providing a resolution to the paradox of histological attaching and effacing lesions that are not associated with inflammatory responses. It also suggests that resident bacteria are recognised by the host and are able to stimulate some of the pro-inflammatory cytokines and other factors in the epithelium, these bacteria may have an important role in the normal physiology of the host intestine.

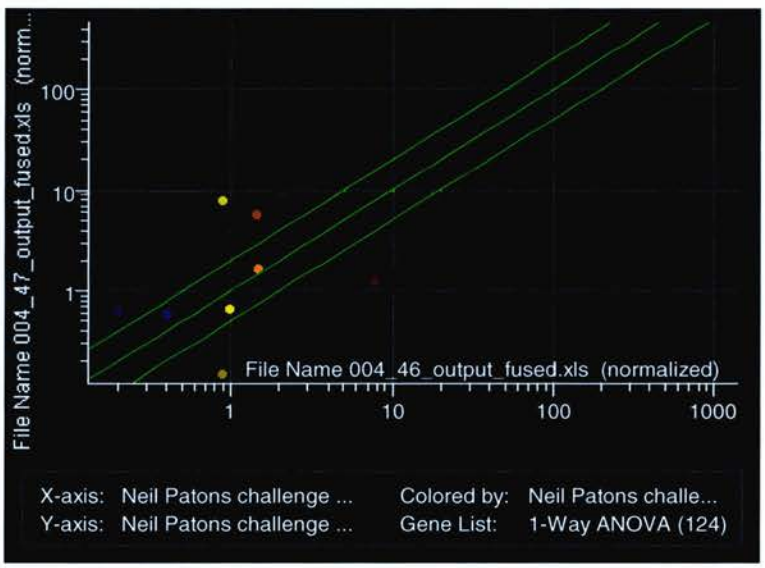
The bovine colon is colonised by numerous serotypes of *E. coli,* the vast majority of which are resident for the life time of the animal and cause no clinical problems. Some of these *E. coli* however are zoonoses and can cause severe pathology within the human host. Most well known of these serotypes is *E. coli* O157:H7 or EHEC which causes diarrhoea, haemorrhagic colitis, and haemolytic ureamic syndrome and can be a cause of mortality in infected patients. Infection of humans occurs following contamination of the food chain, water courses or direct contact with faeces from colonised animals. Several virulence factors are associated with this pathology including Shigatoxin and flagella as well as secreted proteins associated with the LEE Pathogenicity Island. These have been associated with changes to chemokine production, including increases in Il-8 a chemokine that is used to indicate inflammation. EHEC is able to cause attaching and effacing lesions within the host animal suggesting that some or all of these virulence factors are active within the host but there is no evidence for inflammation associated with this colonisation as noted within the host. It was hypothesised that some or all of these virulence factors would be pro-inflammatory within the host and a bovine epithelial model system was chosen to test the pro-inflammatory capacity of the virulence factors within the host. By this method it was

determined that Flagella are major proinflammatory ligands as they produced a profound upregulation in the transcript of Il-8. However other factors have been implicated in the pro-inflammatory response: the presence of the LEE Pathogenicity Island is implicated in the induction of IL-8 transcripts as the strains lacking LEE or LEE components made minor differences in the transcription of IL-8 by EBL cells though work remains to ascertain LEE significance in pro inflammatory response in EBL cells. Shigatoxin despite its profound effects on the human system did not induce IL-8 transcription in EBL cells. However shigatoxin has been shown to have an alternative pathway once bound to Gb3 in the bovine epithelium where it is restricted from its site of action, ribosomes, to the endosomes and lysosomes in bovine cells and may have other roles in the bovine host. ToxB and LifA which are able to influence lymphocytic proliferation and the transport of LEE encoded factors were also examined but change in the transcription of Il-8 by EBL cells was not demonstrated. The model system consisted of epithelial cells and ToxB/LifA exert their effects on lymphocytes; these are within the epithelium although not epithelial cells themselves; more information could possibly be gathered by examining the effects of these proteins on a bovine lymphocyte model system. As it has been demonstrated by others that these proteins limit proliferation of lymphocytes this may act to prevent expansion of the local lymphocyte pool and their ability to clear the bacterium. In addition to EHEC many resident strains of *E.coli* are found in the bovine and some of these were tested against our EBL cells and none were found that increased transcription in a manner similar to EHEC although flagella was present in one strain. Another strain tested which was aflagellate was unable to induce any IL-8 transcription when compared to control levels. Resident bacteria have been utilised in medicine for some time and the most commonly utilised strain is an *E. coli*, termed Nissle 1917, this has been used to treat diarrhoea for some significant time

(Henker et al., 2007) and is beginning to be used in calves (von Buenau R. et al., 2005). Nissle 1917 has a variety of mechanisms for its ability to control diarrhoea including tight junction repair (Zyrek et al., 2007a) and immunostimulation (Hockertz, 1997; Hockertz, 1991) T-cell modulation (Sturm et al., 2005) and defensins induction (Wehkamp et al., 2004) which is mediated by flagellin (Schlee et al., 2007). Although flagellum of EHEC has been demonstrated as a major proinflammatory ligand similar proteins in commensal bacteria fail to demonstrate a pro-inflammatory effect and it is hypothesised in that flagella and TLR5 are separated by intact epithelium and it requires additional pathways to initiate transcription of IL-8, such as disruption of tight junctions. The presence of LEE allows the expression of the type three secretion system and the proteins that are translocated by this system result in changes at the tight junction as factors including EspF influence these host cell structures. Disruption of these structures limits the ability of the cell to control paracellular diffusion and the distribution of cell membrane proteins therefore basolateral proteins including TLR5 will become exposed to intestinal contents including flagella and the pro-inflammatory cascade already outlined will become activated. Resident bacteria in the bovine host may be able to maintain the tight junctions in the bovine and ameliorate this flagellar pro-inflammatory response in a manner similar to the actions of Nissle 1917. Resident bacteria may block or reduce the effect of EHEC on our model system when co-challenged with pathogenic bacteria. Further investigations into the interaction of these and other resident bacteria with the epithelium and EHEC strains are required to characterise the interaction between these three components of a complex system.

Chapter 4.

Microarray analysis of Epithelial cells following colonisation by *E. coli* O157:H7.



A screen image from Genespring^{tm}

4.0 Microarray analysis of epithelial cells following *E.coli* O157:H7 colonisation.

The use of microarray platform technology has exploded in the last 10 years as a means to explore complex systems in biology in an attempt to gain an overview of the complexity within an organism. This approach has been much used in the past to explore the expression of genes within a variety of tumours. It has revealed much about cancer biology for example (Mohr et al., 2004; Kumar et al., 2007; Furusato et al., 2008) and the genes that are expressed or repressed within the cell populations that give rise to tumours. It is only recently that the host/pathogen interface has been explored with this technology and once again it has developed interesting insights in to the molecular communication that occurs between host and pathogen or commensal bacteria (Hayes et al., 2006; Stekel et al., 2005; Mans et al., 2006; Fukushima et al., 2003).

One advantage of microarray technologies is its ability to measure hundreds to thousands of genes in a single assay when compared to the gene by gene approach required by traditional approaches. The technique utilises the base pairing of DNA coupled with fluorescence markers to analyse the expression levels of specific sequences within the mRNA population of a specific experimental sample. At its simplest the RNA is extracted from the cell population of interest and from the relevant control population (e.g. normal vs. cancerous or infected vs. non-infected). This RNA is converted to cDNA by reverse transcription; at this point two fluorescent dyes can be incorporated, Cy5 and Cy3, which fluoresce in the red and far red part of the spectrum, to the DNA molecule being transcribed. The target chip is spotted with the complementary strands of sequences for the genes of interest and the cDNA retrieved from the cells can be hybridised to the gene

sequences bonded to the slide. Laser scanning of the chip induces fluorescence of the spots that have bound genes and allows fluorescence levels to be measured by a Charge Coupled Device (CCD) or similar device. Labelling the two RNA populations with different colour dyes, conventionally referred to by the false colours red and green, allows the relative expression levels for each experimental subject to be compared. This basic approach has been used extensively but requires a relatively abundant source of mRNA from which to produce the cDNA which is hybridised to the array. It also requires significant adjustment to compensate for the limitations of the technology utilised. Where there is little of the source material from which to extract RNA and produce cDNA a means of providing enough material for the microarray must be found. A survey of the literature reveals that the most commonly employed technique is to amplify the source material using one of a range of techniques.

Amplification of the source mRNA is required to produce a usable amount of RNA to allow hybridisation from initially small samples. The appropriate amplification technique is the subject of considerable debate and PCR and linear amplification methods are available to the investigator in amplifying the signal into quantities that are usable within the microarray itself. PCR technology provides an exponential amplification of the collected RNA following tailing of the transcripts with a polyG signal. There would appear to be little to choose between the two approaches however the approach that is usually combined with laser capture as used in this study is linear amplification for which kits have been specifically optimised. The technique utilised is commonly referred to as the Eberwine technique (Van Gelder et al., 1990). This allows the linear amplification of mRNA and there is now suggestion that there is a requirement for at least one round of amplification to improve sensitivity and repeatability of microarray experiments (Feldman et al., 2002). Where there is

little RNA available in the sample as in the case of laser capture material a single round is absolutely required and a second round of amplification is frequently required to achieve sufficient amounts of RNA to allow microarray analysis and this may in fact increase the reliability of the results obtained from the microarray. It was decided to use linear amplification to gain suitable amounts for analysis and to increase the fidelity of the data from the chips.

To examine the response of rectal epithelium to colonisation by *E. coli* O157:H7 32 calves were obtained from local farms. Faecal sampling of the calves was carried out to ensure they were not already colonised by *E.coli* O157:H7. Faeces were diluted in PBS and underwent selective culture on sorbitol plates. This allowed the presence of *E. coli* O157:H7 in the faeces of the calves to be detected and these were removed from the challenge. The cattle were randomly allocated into three groups. The first was a control or unchallenged group of 8 calves (MRI_REF 13, 14, 15 and 28 – 32), two batches of 6 calves were challenged with a naladixic resistant Walla Walla 3 strain of *E. coli* O157:H7 to give the challenge group (MRI_REF 1 – 12). A third group of 12 calves was challenged with a shigatoxin positive strain of Walla Walla 3 but are not used in this study (MRI_REF 16- 27). Daily sampling and culture of faeces was undertaken. This was carried out for each of the calves for each day of the 14 days of the experiment until the calves were sacrificed for the retrieval of the tissue from the recto-anal junction. Following sacrifice some tissue samples were placed in PBS to allow *E. coli* O157:H7 attached to the epithelium to be detected by culture. Tissue pieces 5mm square were washed in PBS and the supernatant was plated out as for the faeces samples. *E. coli* O157:H7 was detected in all the challenged animals (Figure 27) but the control animals remained free from *E. coli* O157:H7 throughout the challenge.

MRI_REF	Average CFU of tissue samples
1	4203.33
2	403.30
3	23.30
4	0
5	43.33
6	0
7	
8	
9	
10	
11	63.30
12	2123.33

Figure 27. Average Colony Forming Units (CFU) from the challenged animals.

MRI_REF numbers 1- 12 were challenged with *E. coli* O157:H7 and the number of bacterium were assessed to give the CFU for each challenged animal as noted above. Calves numbered MRI_REF 13,14 15 and 28 to 32 were unchallenged. The data for the control animals was uniformly negative throughout the challenge.

4.1 Laser capture of epithelial cells

Combined with the ability of the microarray to allow global analysis is the ability to specify the exact cells to be studied; the most popular technique for isolating specific cells is laser capture microdissection, and this has been used in several laboratories (Matsuzaki et al., 2004; Shimamura et al., 2004). This approach combines the specificity for particular cell populations, while allowing the investigation of the overall total response to the interaction with pathogen and other tissues and cell types within the host. In this work the power of the microarray approach has been combined with the ability to select the precise cell population of interest, in this case the epithelial cells. An example of the selection process is provided in the images below (Figures 28, 29 and 30). Although single images are presented here the

microarray data obtained comes from multiple tissue sections. Five histological sections were prepared from each of the six control or challenged experimental animals. The tissues were prepared from the terminal rectum within 3 cm of the recto- anal junction, the main site of colonisation by *E. coli* O157:H7 in cattle. To ensure the bacterium was present at time of sampling bacterial culture of faecal samples prior to post mortem sampling was carried out, in addition tissue samples were taken at time of post mortem for bacterial culture. For the latter a 5mm square section of tissue was vortexed in 5ml of PBS and 100µl of the suspension was plated out in triplicate for bacterial counts (figure 27). Only cattle with a positive culture for *E. coli* O157:H7 over the 14 days duration of the challenge in the tissue sample were utilised for laser capture and microarray. From the histological sections epithelial cells from 5 crypts were captured to give a yield of approximately 5000 cells. Multiple sections were captured on a cap and the total RNA was pooled from these samples. mRNA was extracted from cells obtained from challenged and control animals and amplified for use in the microarray experiments.

Microarray was carried out using a 21000 cDNA array in a dye swap experiment. The microarray compromised 21000 spots of expressed sequence tags within which there are 3 replicates for each spot that represents a gene. cDNA from each of the animals was labelled with both dyes for hybridisations hence 12 slides were used for analysis.

The first image (Fig 28) provided is the intact section image as seen from the microscope. Image colour is due to the lack of cover slip and low power of the light source required to see the laser when cutting. A black line denotes good application of the laser cap plastic membrane ensuring a good adherence to underlying cells and good removal.

Figure 28. A 5um tissue section is prepared for Laser capture.

The lack of coverslip and low light intensity gives a darker colour to the image than that of conventional H+E stained sections. A dark line on the section following application of the laser is indicative of adequate wetting of the PVA layer which adheres to the section and allows removal of specified cells.

This second image (Figure 29.) is of the same section following the removal of the cells of interest. Comparison with the initial image shows the loss of the epithelial cells and the retention on the slide of cells below the epithelium such as those comprising sub-mucosal tissues. This selectivity ensures that a maximal yield of epithelial cells is captured with minimal contamination from other cell types.

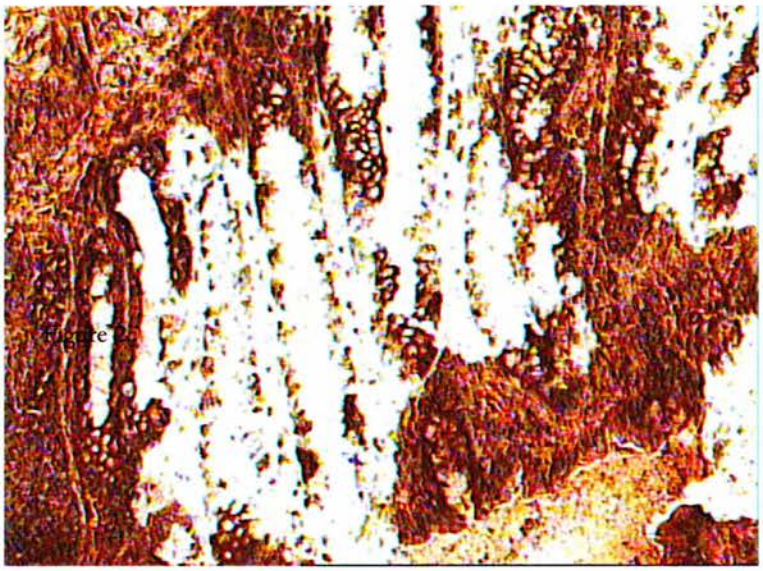


Figure 29. Tissue section post capture section
Following laser wetting of the section seen in figure 2 the cap is removed from the section and adherent cells can are taken with it. The section here demonstrates the techniques ability to remove the epithelial cells of the crypt and leave the cells of the deeper tissues in situ. The White areas are where the cells have been removed.

The final image (Figure 30) is that of the epithelial cells that adhere to the cap. Cells from several locations can be identified as several areas can be captured on the same cap. The amount of cells from each section was highly variable but 4000 - 7000 per section were usually available, the number of cells available was limited by the orientation and size of the section. In order to get the best orientation and allow the collection of the largest number of cells the samples were frozen in a specific manner: the mucosal surface of each tissue block was trimmed of excess fat and placed on a brass plug. This plug was lowered into a bath of liquid nitrogen. This provided rapid controlled freezing and samples could be held at -70^{0}C until required. The brass plug also gave a flat surface that could be orientated on the cryostat. An acceptable cross section gives the maximum chance of a good capture of cells.

Figure 30.Cells adherent to the cap following capture.

Adherent material from the cap can be identified as representing the cells that are of interest. If required sections that have too many contaminating cells can be discarded. Only a small section of the cap is shown and far more cells than this can be captured per section.

4.2 Proteomic analysis of laser captured material

Cells captured from the sections were used to examine the proteins extracted by the technique. Following the protocol outlined in the Material and Methods chapter proteins were separated by SDS PAGE, bands resulting from coomassie blue staining were cut out and proteins were extracted. The extracted proteins were analysed by MALDI-TOF analysis at the Moredun Proteomics Facility. Few proteins were observed although Bovine Cytokeratin 19 was identified as being present in the sample (expectation value 0.001, score 83). This protein is epithelial specific (Hamakawa et al., 1998; Pujol et al., 1993; Miedouge et al., 2001) which gives support to the epithelial nature of the cells that have been captured

however this does not guarantee the purity of the captured cells. As no other proteins were identified in this analysis no further conclusions can be drawn especially as differences between the protein levels were not determined. However this does suggest an area of possible further investigation as the presence of specific markers for colonisation can be investigated. If combined with the latest LCM equipment, which can isolate cellular compartments such as mitochondria, proteomics can possibly detect specific components. The small amount of material presents a massive technological challenge to this approach.

4.3 Analysis of microarray data

As might be expected the data that is produced from a laborious process such as this requires a thorough and rigorous approach to the analysis. The initial data capture step is the analysis of the images generated by the scanning process. This was carried out using Bluefuse software (BlueGnome™). The software allows the delineation of spots and the removal of artefacts such as dust spots. Image intensity is then measured at different levels and the best exposure is determined; the CCD detectors that capture the light emitted from the spots have defined detection limits, too low fluorescence and they will not be able to detect a signal, too high and the CCD will be swamped and record a measurement at the maximum value of the detectors limit despite the intensity of the spot being much higher. These two extremes represent a loss of potential information and most current software is not able to combine the data produced. Therefore a compromise setting is usually employed, which allows the capture of the largest amount of meaningful data. However this raw data cannot be interpreted without further mathematical operations being carried out as the linear

relationship that might be expected breaks down at both the very low and very high fluorescence levels (Figure 31).

This breakdown in the relationship of the data has lead to the development of several techniques that propose to return the data to a linear relationship. There are several methods of normalisation that have been utilised. These include adding an exogenous standard, the use of housekeeping genes and the development of mathematical operations to model the actual relationship of the data. The use of housekeeper and exogenous controls gives a known fluorescence/expression level relationship but is still unable to account for the signal compression that occurs and any transfer of energy that occurs between the dyes molecules.

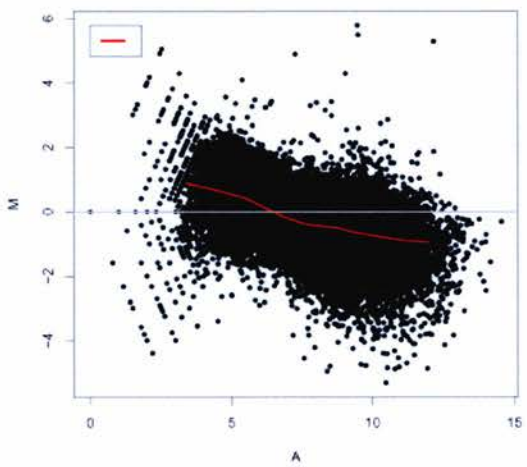


Figure 31.Example of an unnormalised microarray plot.

The red line represents the line of best fit for the data when the Log ratio, M is plotted against intensity, A and is the line where there is no change in the expression level of the genes. Normalisation is used to graph the data as a log ratio of 0 which allows more reliable interpretation. Unnormalised data is non-linear especially at the extremes. Image obtained from Stanford University website http://genome-www5.stanford.edu/help/results_normalization.shtml

The most common approach to normalise the data within arrays is the use of normalisation algorithms. These algorithms are commonly incorporated in the software packages and a Global loess normalisation algorithm is included in the Genespring™ (Silicon Genetics) software package. This algorithm, and other related mathematical techniques, are now the most common approach utilised for normalisation (Kreil and Russell, 2005; Listgarten et al., 2003). The raw data from the chip does not follow a linear relationship as might be expected with an increased level of expression not giving a level of fluorescent imaging that is directly proportional to the target signal. This phenomenon is referred to as signal compression. Light emitted from each spot can be altered due to the free energy transfer between the dye molecules as well as the hardware factors discussed. Normalisation attempts to correct for these factors by deriving the curve through small groups of data. The algorithm empirically derives a correction that allows this curve to be returned to the linear 1:1 ratio that is required for further analysis. This is carried out as an automatic operation following loading of the data into the Genespring™ platform.

Once the data was normalised, the signal intensity of genes represented by the spots were subjected to ANOVA analysis to allow the genes that were differentially expressed with a statistically significant degree of difference to be identified. This removes genes from the analysis where there is too little data to allow certainty with regard to the expression level. Expression levels are usually expressed as fold changes, as either 2 fold upregulated or 2 fold down regulated are considered the minimum fold change for further analysis. This fold change cut off while arbitrary is commonly used in this type of analysis. From these criteria two lists were generated for both up (figure 32) and down (figure 33) regulated

P-value	NCBI Blast result	GENE ID	BLAST SCORE	Possible function	Fold Change
0.0329	XM_584969	Zinc finger protein 161	589	G2/M cell cycle transition inhibition	4.5
0.00659	XM_877124	CG8202-PA	36.2	?????	2.7
0.0248	BC103419	Bos taurus cDNA clone MGC:127919	591	Cell cytoskeleton	2
0.0049	XM_609564	Bos taurus similar to Toll-interleukin 1 receptor	684	toll signalling pathway linked to myD88	2.5
0.00507	NP_006816.2	cytoskeleton-associated protein 4	N/A		2.9
0.0167	XM_594564	Bos taurus similar to Est1p-like protein A	1114	teloromase maintenance	2
0.0067	N/A	Glutathione peroxidase 4 (phospholipid hydroperoxidase)	N/A	response to stress	2.1
0.0162	XP_522454.1	glucosamine (N-acetyl)-6-sulfatase		keratin metabolism	2.1
0.0347	CO888540	KIAA1737 protein		Possible alternate gene has role in rna posttranslational modification	2
0.00864	XM_873777	Bos taurus similar to cadherin 10	38.2	cell adhesion	2.6
0.0134	NM_174669	Bos taurus serine (or cysteine) proteinase inhibitor	3326	C1 inhibitor	2.2
0.0331	NP_000080.1	collagen, type I	N/A		2.3
0.0133	CO879541	*Fibronectin 1*	N/A	cell adhesion wound healing	2

Figure 32. Up regulated genes.
Following ANOVA there were 14 genes identified as being up regulated these are listed here. The gene name is the EST sequence identity from the array data. P-values indicated are those that were derived from the ANOVA testing suggesting that the changes are real but multiple correction testing was not carried out in this data. NCBI derived gene ID and function are also presented here.

Gene Name	P-value	Description	Blast score	NCBI ID	Possible function	Fold Change
C0005199n3	1.94E-05	Bos taurus similar to CG17660-PA	36.2	XM_881726	transmembrane receptor	
Spotting Buffer	0.002					
CO896833	*0.0135*	*Bos taurus similar to ATP-dependent RNA helicase ROK1*	*79.8*	*XM_588033*	*rna processing*	*6.3*
CO896955	*0.0142*	*Bos taurus similar to CG15792-PA*	*34.2*	*XM_616238*	*Cell division*	*2.3*
CO888571	*0.0288*	*G protein-coupled receptor, family C, group 5, member B*	*N/A*	*NP_057319.1*	*anti apoptosis*	*2.3*
CO880713	0.00585	Bos taurus clone ep42-152a4, complete sequence	404	AC096629	unknown	5.6
CO878409	0.0292	Bos taurus BAC CH240-243M2	105	AC150687	unknown	*1.8*
CO896860	*0.00661*	*Bos taurus similar to Cyclin C*	*212*	*BC102107*	*Cell division*	*6.1*
CO874006	0.0182	Linked to web page saved under gene name	32.2	AC150516 DP000008 AF401289		*2.6*
CO878067	*0.0351*	*similar to nucleoporin 214kDa; nuclear pore complex protein Nup214; CAN protein, putative oncogene; p250; nucleoporin 214kD (CAIN)*	*N/A*	*XP_520324.1*	*Cell division*	*2*
C0006015a02	0.0439	PREDICTED: Bos taurus similar to CG5290-PA PREDICTED: Bos taurus similar to Prominin 1 precursor (Prominin-like protein 1) (Antigen AC133) (CD133 antigen)	36.2	XM_603246 XM_870384	Prominin is epithelial marker and locates in microvilli,	2.6
CO881110	0.0341	similar to GB|AAA02600.1|185871|HUMIGKAMI immunoglobulin kappa-chain {Homo sapiens;	N/A	N/A	Dendritic cell marker?????	2.2
CO883312	0.033	Bos taurus target 1 genomic scaffold.	38.4	DP000008	?????	*2.1*
CO879944	0.0434	PREDICTED: Bos taurus similar to Alcohol dehydrogenase 6 PREDICTED: Bos taurus similar to mitochondrial ribosomal protein L53, transcript variant 3 (LOC505728), mRNA. PREDICTED: Bos taurus tyrosine kinase receptor (flk-1), partial mRNA.	36.2	XM_587704 XM_873850 XM_612264		2.1
C0005203d16	0.0338	PREDICTED: Bos taurus similar to sulfatase 2 isoform a precursor, transcript variant 1 (LOC533264), mRNA.	1362	XM_612618	heparin sulphate proteoglycan metabolism	2.2
CO885817	0.0321	Linked to web page saved under gene name				2.7
CO884238	*0.0313*	*jumonji domain containing 2B*	*N/A*	*NP_055830.1*	*possible transcription regulation*	*2*
CO878909	*0.0182*	*homologue to GB|AAH47950.1|28839621|BC047950 TTC9 protein {Homo sapiens}, partial (5%*			*Cell division*	*2*
CO873005	0.0387	Glial fibrillary acidic protein			structural protein	2
C0005204h5	0.0237	Multiple possible homologies				2.1
CO871904	0.0123	PREDICTED: Bos taurus similar to ribosomal protein S21 (LOC615178), mRNA.	646	XM_866914		2.1
C0005202k19	0.0159	Bos taurus Toll-like receptor 10 mRNA, partial cds.	65.9	AY634632		2
CO875027	*0.0192*	*Transcribed locus, moderately similar to XP_525295.1 similar to Soggy-1 protein precursor (SGY-1) (UNQ735/PRO1429) [Pan troglodytes]*	*N/A*		*embryonic development*	*2*
CO880080	0.00282	homologue to UP|Q948Y7 (Q948Y7) VMP3 protein, partial (3%)			protease ???????	2
CO878308	0.0322	Purinergic receptor P2X, ligand-gated ion channel, 4			atp involved signalling	2
CO873902	0.0354	Transcribed locus, strongly similar to XP_527786.1 similar to Williams Beuren syndrome chromosome region 21 isoform 2 [Pan troglodytes]			Unknown function but related to brain dysfunction in humans following deletion	2.4
CO887911	0.0276	PREDICTED: Bos taurus similar to olfactory receptor, family 8, subfamily B, member 3 (LOC506960), mRNA PREDICTED: Bos taurus similar to F-box and leucine-rich repeat protein 15 (LOC519874), mRNA	34.2	XM_583490 XM_598105	g-coupled receptor possible, ubiquitnastion of proteins	2
CO884814	0.000259	Solute carrier family 25 (mitochondrial carrier; oxoglutarate carrier), member 11			Glutathione transport activity	2
C0006018a21	*0.0118*	*PREDICTED: Bos taurus angiopoietin 1 (ANGPT1), mRNA.*	*40.1*	*XM_612513*	*Blood vessel growth*	*2*
CO878468	*0.0164*	*PREDICTED: Bos taurus similar to angiopoietin-like 1 precursor, transcript variant 3 (LOC509971), mRNA*	*38.2*	*XM_879258*	*expressed in all highly vascular tissues*	*2.1*
CO894293	*0.0451*	*PREDICTED: Bos taurus similar to 5-nucleotidase, cytosolic III, transcript variant 1 (LOC511449), mRNA.*	*486*	*XM_588784*	*pyrimidine nucleoside metabolism*	*2*
CO894567	0.0229	similar to UP|Q9XLN5 (Q9XLN5) ATP synthase subunit 6 (Fragment), partial (9%)				*2.3*
CO879358	0.0494	Linked to web page saved under gene name				*3.6*
CO874290	0.0169	similar to UP|Q9XSA0 (Q9XSA0) Pulmonary surfactant-associated protein B (Fragment), partial (38%)				2.2

Figure 33.Down regulated genes on the Array.

Following ANOVA there were 32 genes identified as being down regulated these are listed here. The gene name is the EST sequence identity from the array data. P-values indicated are those that were derived from the ANOVA testing suggesting that the changes are real but multiple correction testing was not carried out in this data. NCBI derived gene ID and function are also presented here.

However a more rational method to look at this may have been to look at intrinsic variation within the array data. The fold change represented is a summary of the levels of a multiple of spots on a number of microarray slides. The range of values across these may accurately represent the intrinsic range found in the population of animals or experimental replicates. By examining this value for genes that are considered unchanged, genes that are sometimes referred to as housekeeper genes, a conclusion can be reached as to the intrinsic variation in the experimental system. We could therefore use this figure, which will vary from system to system, to identify those genes that may be differentially expressed at levels greater than that expected through intrinsic variation. There are many other criteria that could be applied to the data derived to include or exclude specific genes, however as previously stated to be in line with current literature the 2-fold cut off approach was taken.

These lists (up and down regulated) were initially populated with the reference numbers for the EST sequences archived in the NCBI database that represent the Cdna sequences derived from the brain/spleen library that were used to print the chip. These sequences were used to run a BLAST search using the NCBI site application to find matches The data derived gave a variety of expectation values (e-values) and scores and the most likely identity (Lowest e-value) was used as the putative identity. The score was also considered but this tended to follow the e-value so did not influence the choice of target assigned. The identification information was used to conduct a literature search to define possible roles for identified factors in the colonisation of *E. coli* O157:H7. The literature search using the lists of genes generated allowed a rationale to be developed that allowed the expressed genes to be grouped into logical sub-groupings by taking into account biological function. Analysis of NCBI databases once again produced a wealth of information and the

cross database search facility was used to mine the vast amount of information that exists for the differentially expressed genes within the literature. This information allowed a prediction for the role of the genes expressed in EHEC colonisation to be made with genes arranged into biologically relevant groups, the numerically largest being a group with confirmed or possible involvement in the proliferation of cells. The choice of targets for further analysis was Zinc finger protein 161, Jumonji domain containing protein 1B, Est-1p like protein, Cyclin C, and Angiopoietin-1 like protein. qtPCR technologies were used to validate the results and a phenotypic assay to be chosen to confirm the hypothesis derived from the data (See next chapter).

Additionally a FASTA search was conducted on sequences that were considered of further interest, using sequences published for proteins that have possible interactions with those previously identified on the up regulated or down regulated lists. This allowed us to identify possible differential regulation of genes that may be below the 2 fold cut off point, for example Zinc finger protein has been identified as associated with Zinc finger 295 (Wang et al., 2005). The sequence for this was identified from public databases and applied in the FASTA algorithm for possible EST sequence matches. FASTA analysis was carried out as the algorithm attempts to match short sequences (words) between the sequences of interest and the database sequences, in this case the EST sequences of the array dataset. This returned an EST identity which was examined in Genespring^{tm}. Changes in gene expression were noted from the software and this information and the genes identified in this data mining approach are listed in the following table (Figure 34). FASTA analysis of the EST database from Ark genomics was carried out by A Grosvener (Edinburgh University). The changes in gene expression may be significant in the biology although with current analysis

techniques it is difficult to ascertain their role in pathogenesis and further validation of these targets is required.

Gene Name	EST sequence identifier	Score	e-value	Median Fold change
Retinoblastoma	CO893853 CO874898 CO887792	34	1.60E-01	1.047
CDK3	CO888546	186	2.00E-46	0.79
cdk8	CO886509	34	1.10E+00	0.833
c-myc	CO888852	34	2.70E-01	0.856
RNA polymerase II	CO892025	34	1.90E+00	0.8395
Cyclin D	CO891827	36	1.90E-01	0.237
Cyclin D		34	7.60E-01	
TIE-2	CO879117	34	7.40E-01	1.14
VEGF	CO878568	34	5.70E-01	0.527
PI3 Kinase	CO892033 CO889788	36	5.70E-01	1.29
Caspase 7	CO891998	34	8.30E-01	0.837
Caspase 7	CO886279	34	8.30E-01	1.02
Caspase 3	CO887288	34	1.70E+00	1.028
Caspase 9	CO881702	34	1.50E+00	1.164
FAS	CO881606	321	5.00E-87	0.904
VEGFR	CO882974	36	6.30E-01	0.986
SMG1	CO894334	38	4.90E-01	0.98
UPF	CO895700	38	2.30E-01	0.944
Est 1b	CO886090	38	1.80E-01	1.3
Zinc finger 295	CO888162	38	0.14	1.11
Zinc finger 295	CO880671	38	1.40E-01	
TERT	CO882030	36	4.90E-01	up regulated
EST1A	CO883187	32	7.40E-01	1.055
INTERSEX	CO891106	36	5.60E-01	0.8465

Figure 34.Genes of interest.
These were identified from literature search relating to the group of genes that were chosen for validation. The sequences from the NCBI database were compared to the EST sequences on the array using the FASTA search algorithm. This gave a possible spot correlation for each of these genes. This allowed some information to be derived from the array about the pathways that we speculated might be involved in the alteration of these 6 genes. The identity frequently did not rise to a level where confidence could be established with the exception of CDK3 and FAS. The Median fold change from the data on the array is listed with none showing a greater than 2-fold change. This means that we cannot conclude with certainty that any of these genes are directly differentially regulated. However this does not preclude the change in state of these proteins being involved in the pathway rather than a change in expression level.

The FASTA search was able to identify very few genes that might be involved in the pathways, however it was able to correctly identify the genes already listed in tables 32 and 33 so there is confidence that this is a robust approach in identifying the genes that may be involved in the pathways. The targets identified in the FASTA search are potential candidates for further validation. These targets may have a fold change below 2 which has

been widely used as a cut off for differential regulation but these targets may function in the pathway after activation by such actions as phosphorylation. The array technology used in this work detects changes in gene expression but is unable to detect changes in activation. Validation of these targets requires careful design of experiments to detect the relevant changes.

To go forward for further investigation the genes selected needed to fulfil two requirements. It had to be differentially regulated by greater than 2 fold and there had to be a biological rationale for it to be included in a group. Once the identity of the gene was ascertained a literature search was undertaken to find its biological role. As the largest biological grouping of genes was that with a suggested role in the cell cycle and there is literature evidence to support an influence by *E. coli* O157:H7 on proliferation of cells this group was selected for further investigation. The genes to be studied further are Zinc Finger protein 161, Est-1p like protein, Jumonji domain containing protein 2B, Angiopoetin like protein 2B and CyclinC. These will be used in quantitative Real time PCR for validation of the array data and further experiments in an attempt to validate any putative phenotype.

Chapter 5

Influence of *E. coli* O157:H7 colonisation on epithelial cell proliferation.

Recto-anal junction (H+E stain)

5.0 Influence of *E. coli* O157:H7 colonisation on epithelial cell proliferation.

The most fundamental function of the intestine is closely linked to its anatomy and its physiology, not least that of the epithelial surface lining the length of the intestinal tract. Among the most characteristic feature are the villi, which are finger like projections into the lumen of the gut. The epithelial cells of these structures have a further folding of the surface into microvilli and both of these adaptations assist in increasing the surface area for absorption of nutrients and control of fluid balance. The functions of these cells are tightly controlled by a large number of signalling cascades, with many stimuli such as mitogens, anti-apoptotic signals and cell-cell contact signals controlling proliferation and differentiation (Matter et al., 2005a; Clatworthy and Subramanian, 2001). In addition these cells are exposed to micro-organisms including potential pathogens that may enter the lumen of the gut. Therefore epithelium serves in an immune surveillance role being involved in the initial response within the lumen to pathogens. The gut has a number of mechanisms for the removal of pathogens in the gut of which two are peristalsis and mucus production by Goblet cells, with mucus inhibiting and binding bacteria which allows peristalsis to move the bacterial load down the intestinal tract. In addition cell death and apoptosis removes intracellular and intimately attached pathogens, by the loss of infected cells before bacteria can spread throughout the intestine or into deeper tissues. More highly specialised cells within the epithelium - including M-cells - are involved in the control of the adaptive immune responses (Hathaway and Kraehenbuhl, 2000; Kraehenbuhl and Neutra, 2000).

A large number of sites within the host are colonised by bacteria and, to a lesser extent, other micro-organisms. All external and mucosal surfaces are populated by colonising

organisms; the intestine is the most highly populated site with the large intestine home to at least 10^{11} bacteria per gram of faeces thus a huge variety of bacteria have adapted to the niche that is the anaerobic environment of the intestine. Consequently, the host is adapted to the presence of these bacteria such that disease does not result; indeed many of these micro-organisms and their host organisms have a mutual dependence on each other (Gordon et al., 1997). The development of the intestine from the neonate requires the colonisation by normal faecal flora otherwise abnormal development of the intestinal anatomy, physiological function and immunity occurs (Hooper et al., 2001; Hooper, 2004); for example normal motility requires the presence of the normal bacteria flora as does angiogenesis in the intestine. Additionally bacteria can affect the host as an adult and maintain homeostasis of the epithelium including the ability to affect cellular proliferation.

Therefore the interaction of bacteria with the intestine is a normal process however the interaction of the epithelium and bacterium is not always benign and pathological changes occur. Antimicrobial peptides and chemokines may be induced in response to the colonisation by bacteria. Pathogens may induce the production of antimicrobial peptides (Bals, 2000) which may control the level of bacteria in the host. The production of chemokines is part of the communication within the host that allows normal homeostasis within the epithelium(Rimoldi et al., 2005) and involves communication within many cell types in the epithelium. (Nishiyama et al., 2002). However bacteria are also influencing epithelial cells by communicating with these cells (Clavel and Haller, 2007; Gordon et al., 1997) and the response to these signals can lead to pathological changes within the host.

EHEC colonises cattle without causing clinical disease. EHEC is predominantly a human pathogen where it can have fatal consequences (Kaper et al., 2004) . Indeed, in

humans it induces diarrhoea, in the majority of patients, and, in a small number of patients, more severe consequences such as haemorrhagic colitis, haemolytic ureamic syndrome and other associated syndromes. A very small proportion of patients will have chronic renal conditions and a small number will suffer fatalities. The more significant of these conditions are due to the presence of Shiga-like toxin (Stx), which binds to Gb3 receptors on microvascular endothelial cells present in disparate regions such as the intestine and renal glomeruli (van Setten et al., 1997; Robinson et al., 1995). In susceptible patients, this can lead to apoptosis of endothelial cells and consequent loss of fluid control across intestinal epithelium with loss of blood from the circulation into the urine and faeces respectively. Thus, death in humans is mainly a consequence of the loss of the ability to control fluid loss and the resulting profound dehydration although there are other systemic and endothelial events that contribute to fatalities. As already mentioned, these bacteria tend to asymptomatically colonise cattle although they can cause histological lesions in the epithelium of cattle (Stordeur et al., 2000; Dean-Nystrom et al., 1999) with resultant clinical disease, although the animals concerned tend to have some form of immunocompromise (colostrum deprived etc). EHEC has a wide variety of virulence factors that would be expected to compromise host cell viability as is seen in the human, however cattle appear to tolerate colonisation and A/E lesion formation does not appear to alter the function of the epithelium as a whole. This suggests that, in contrast to human infections, *E. coli* O157:H7 may allow greater colonisation of this niche through manipulation of the intestinal environment towards allowing a greater period of colonisation within the reservoir host. This may be by the inhibition of inflammatory responses, cell mediated immunity or inhibition of cell apoptosis within the epithelium.

As described in the previous chapter, normal cattle were challenged with a Shigatoxin negative EHEC O157:H7 (strain Walla Walla 3) until peak colonisation was achieved at around day 10-14 post-challenge. Rectal tissue was sampled and epithelial cells were isolated using laser capture microdissection from which RNA was extracted for analysis using a cDNA microarray approach. The data from this approach allowed identification of groups of bovine epithelial cell genes that appear to be involved in the response to colonisation by *E. coli* O157:H7. The largest group that could be discerned was apparently involved in the regulation of the cell cycle. From the data obtained several of these - Jumonji containing protein 2B, zinc finger protein 161, Angiopoietin-like 1 protein, Cyclin C and EST-1p-like protein – were chosen for further investigation as data suggested these acted as part of closely related pathways. As presented in the microarray assessment chapter these proteins all converge on Retinoblastoma protein (pRb), a critical cell cycle "gatekeeper" protein for which function is controlled by (de)phosphorylation, a process that allows release of the transcription factor E2F, which induces the transcription of proteins required for cell division (Allen et al., 1997; Francesconi et al., 2000; Ikeda et al., 1996). The data obtained suggested that the expression of these proteins is up-regulated following colonisation by EHEC and changes in the regulation of these genes will result in altered cellular replication rates. The hypothesis generated from the data suggested that *E. coli* O157:H7 may be able to alter the proliferation rate of intestinal epithelium. To confirm this, Proliferating Cell Nuclear Antigen (PCNA) was utilised as a cellular proliferation marker, which demonstrated that the rate of epithelial cell proliferation of colonised animals was less than that of the unchallenged animal. The effect of colonisation on pRb phosphorylation and location was examined by an immunohistochemical method; the presence of Rb was

noted in the cytoplasm away from the site nucleus where it is presumed to exert its control of proliferation. This may be consistent with the reduction of proliferation of epithelial cells.

5.1 Results

In the previous Chapter, microarray analysis of bovine epithelial responses to colonisation with *E. coli* O157:H7 identified significant up- and down-regulation of 13 and 34 transcripts respectively which had a fold change increase or decrease of greater than 2. Further analysis was carried out to confirm expression changes in a further subset of these genes.

5.1.1 qtRT-PCR analysis of gene expression in the rectal epithelial cells.

5.1.1.1 Housekeeper gene.

From the microarray analysis, a gene was identified that did not show an increase or decrease in the majority of the hybridisations (5 out of 6 arrays). This gene was identified as DNA helicase ATP subunit 70 by BLAST analysis of the EST sequence in the Ark genomics database. Its identity is not considered significant as it was used to identify any technical bias in the amount of mRNA in the experiment. Analysis of the challenged group by real time PCR revealed that this target was expressed at a lower range in the challenged group in comparison to the control group (figure 35) but not significantly lower. This suggested that there was a technical bias to the real time results. To remove this bias we derived a correction factor from the median value to normalise the data. For each of the three replicates per animal an average value was calculated and these values produced a median for the group as a whole. To correct for biases in the experimental system when assayed by Qt RTPCR including differences in transcription and number of cells originally captured a the ratio from the normalisation was calculated.. This gave a correction factor that was applied to all further genes examined, by experimental values by this factor. Each animal was given a

ratio to reflect its difference from the median. This was used on all further values to normalise the two groups. The primers were designed to cross intron-exon boundaries.

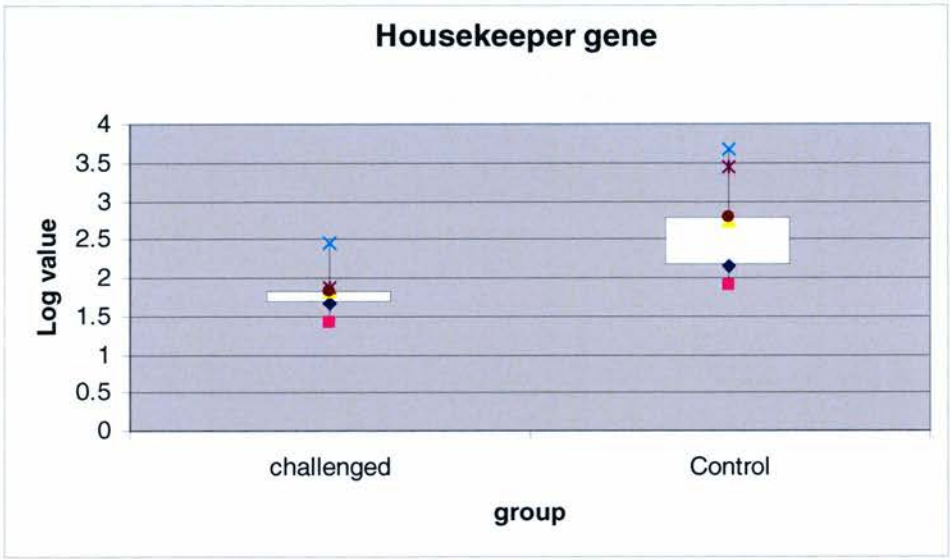


Figure 35.ATP subunit 70 for normalisation.

This gene was unchanged on the array across 5/6 arrays. Comparison of the groups (control and challenged), using a qtPCR analysis, suggested that the control gave a higher copy number group number for this gene hence the median values for each group was used as a correction factor to remove this bias. The transcript level was measured 3 times for each of 6 animals in both groups. Values are represented as $\log_{10}$ transcripts. Differences were assessed by students T-Test. ($p=0.09$)

5.1.1.2 Cyclin C

Cyclin C is a cell cycle regulatory protein that has been shown to mediate cell cycle entry from G_0 to G_1 by activation of Cyclin dependent kinase 3 (CDK3) acting as a physiological regulator of entry into the cell cycle (Ren and Rollins, 2004). Cyclin C forms complexes with CDK3, CDK8 and RNA polymerase II to activate transcription and provide temporal control of the cell cycle (Johnson and Walker, 1999). Cyclin C is therefore a key regulator of cell proliferation. Primers derived from the sequence of the EST used on the array were used for qtRT-PCR, the primers were designed to cross intron-exon boundaries. After normalisation, the median relative transcript levels were 5.7 and 4.2 (Fig 36) and the assay presented here suggests that this gene product is up-regulated within the epithelial cells of cattle colonised by *E. coli* O157:H7.

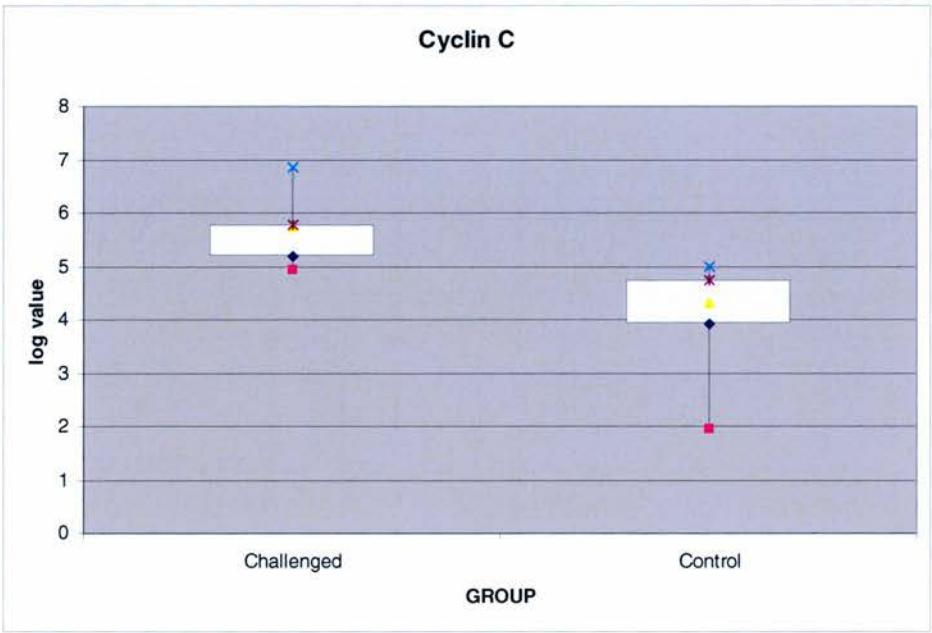


Figure 36. Cyclin C quantitative results

The EST for Cyclin C was used to design a qtPCR assay which was used to assay the copy number of transcript in the epithelium of the rectal mucosa and the Log_{10} for each was calculated. 3 values were derived for each of the 6 animals in each group. The groups were normalised to the housekeeper gene and interquartile ranges were calculated. Cyclin C appears to be expressed in greater quantity in response to colonisation by *E.coli* O157:H7 ($p=0.02$). This change appears to be a 1.2 fold change between median values.

5.1.1.3 Zinc finger 161

Zinc Finger 161 is a known regulator of transcription related to a family of gut localised transcription factors, the kruppel-like transcription factors (Shie et al., 2000; Garrett-Sinha et al., 1996). It modulates transcription within the gut where it acts in combination with other Zinc Finger and Kruppel factors. Primers derived from the sequence of the EST used on the array were used for qtRT-PCR; the primers were designed to cross intron-exon boundaries. In the group of animals colonised by *E. coli* O157:H7 there was a slight difference in the transcript copy number (figure 37) when compared to non-colonised controls although this was not significant when data was analysed by Students t-test (p=0.38).

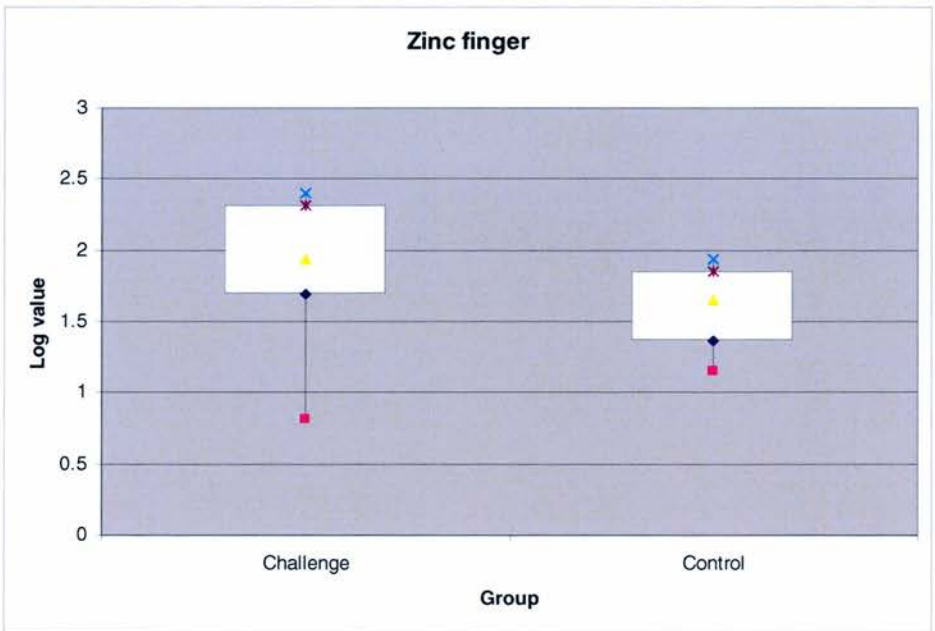


Figure 37. Zinc finger 161 quantative results.
A repressive transcription factor, Zinc finger 161, identified on the microarray was assayed using qtRTPCR 3 replicates from each of the 6 animals was obtained and log values were calculated and normalised against the housekeeping gene. Comparison of the log normalised copy number for Zinc finger 161 demonstrated a fold change decrease of 1.12 but this is not a significant change (p=0.38).

5.1.1.4 Jumonji domain containing protein 2B

Jumonji domain containing protein 2B is a negative regulator of transcription as shown by analysis of murine cardiac muscle cells (Toyoda et al., 2003) (Toyoda et al., 2000; Jung et al., 2005). Regulation of transcription by Jumonji domain containing protein 2B involves interaction with the retinoblastoma protein through cyclinD. Jumonji is localised to the nucleus as it contains within its structure a nuclear localisation domain. Following the interaction of this domain with the DNA transcription repression occurs. In cardiomyocytes this appears to cause repression of CyclinD1 which interacts with a key protein in the cell cycle, retinoblastoma protein. As there was a change in the expression whan examined by the microarray and there was a biological rationale studying the target in more detail a real time assay was designed to further validate the transcript.

Primers derived from the sequence of the EST used on the array were used for qtPCR, the primers were designed to cross intron-exon boundaries. Figure 38 shows that the challenged group expressed this transcript at a higher level than in the control group. The data from this assay suggests that this transcript may be altered by colonisation with *E. coli* O157:H7.

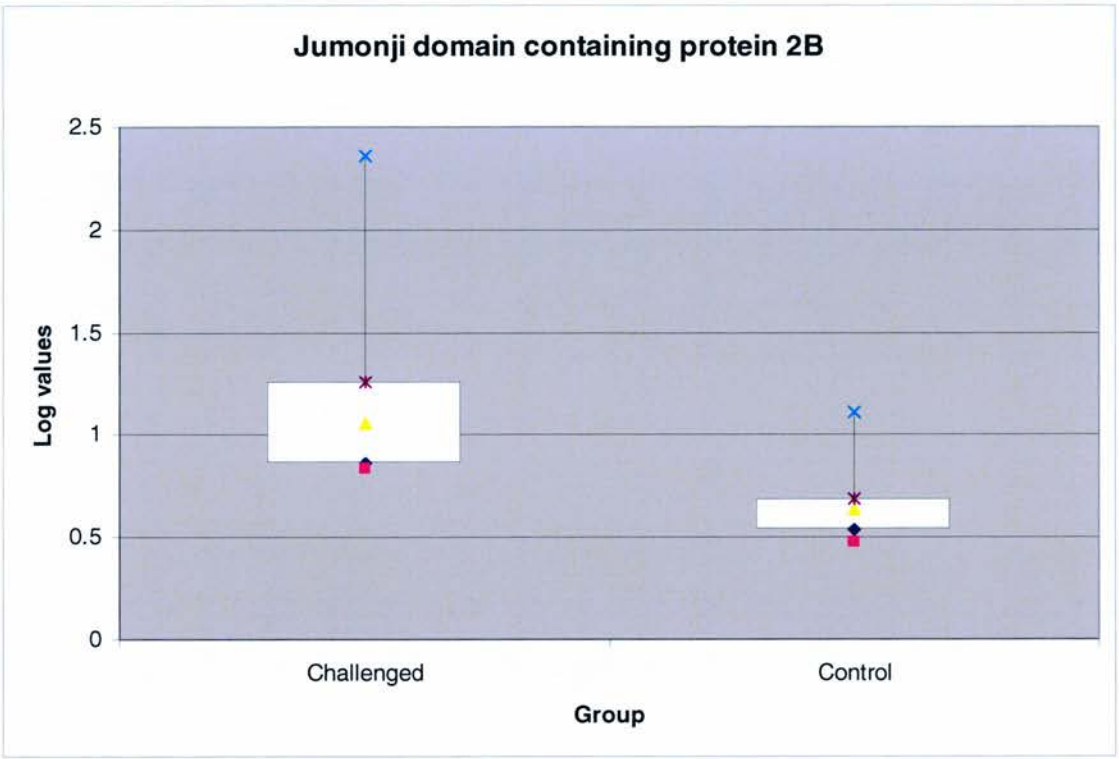


Figure 38. Jumonji domain containing protein 2B quantitative results

qtPCR was used to determine the copy number within the rectal epithelium of challenged and unchallenged animals. The graph is the composite of 3 replicates from each of six animals in the group expressed as a $\log_{10}$.This target is a negative regulator of the proliferation and is upregulated within this system ($p=0.05$). The increase expression of Jumonji is suggestive that there is 1.6 fold change with Jumonji increasing in the epithelium of colonised cattle.

5.1.1.5 Est-1p like protein.

Est-1p like protein is involved in regulating the function of telomerase enzyme TERT (telomerase reverse transcriptase) which maintains the telomeres of cells (Taggart and Zakian, 2003). These accessory proteins either recruit or activate TERT and Telomere maintenance is essential in allowing cell survival through replication. Escape from control by these proteins can lead to immortalisation and oncogenesis.

Primers derived from the sequence of the EST used on the array were used for qtPCR, the primers were designed to cross intron-exon boundaries. The upregulation of Est-1p like protein (fig 39) may point to an increase in cell cycle events within colonised epithelium.

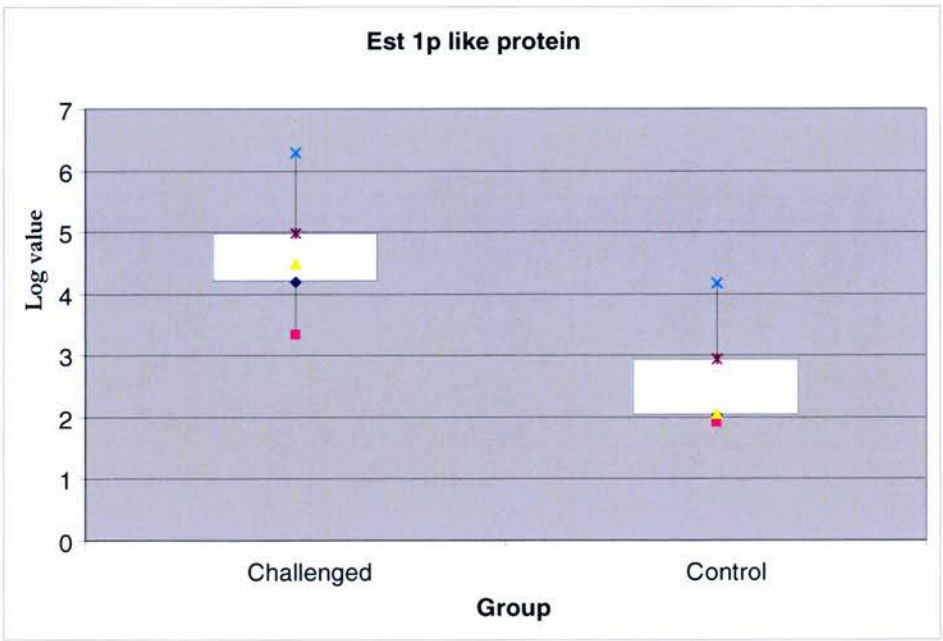


Figure 39. Est-1p like protein quantitative analysis.

Est-1p like protein was assayed by the qtPCR method. Data was normalised to the housekeeper gene. Copy numbers for 3 replicates from each of the animals was found and the data is presented as a $\log_{10}$ value and these values for Est-1p like protein shows that the transcript number in the challenged group demonstrated a 2.24 fold increase in the challenged group which was significant ($p=0.01$).

5.1.1.6 Angiopoietin-1 like protein

Angiopoetin-1 like protein was identified on the microarray as a transcript that was downregulated. Literature searches suggested that there were a number of roles for this protein; these included pro and anti inflammatory roles, both of which could have significance in the pathogenesis of *E. coli* O157:H7 colonisation(Gamble et al., 2000). In addition inhibition of cell apoptosis was noted as a possible role for this protein(Harfouche et al., 2002) and it is known to be mitogenic through a MAPK associated pathway(Kanda et al., 2005). These roles were considered sufficiently interesting to justify the development of a real time PCR to test the microarray data. The transcript levels detected were significantly elevated in colonised animals compared to control animals (Fig 40). Primers derived from the sequence of the EST used on the array were used for qtRT-PCR; the primers were designed to cross intron-exon boundaries.

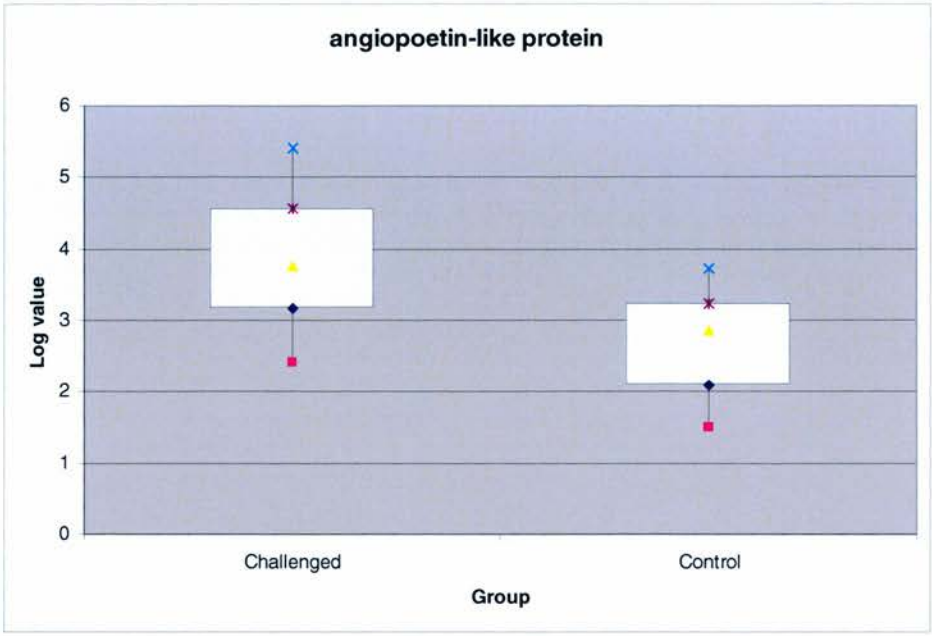


Figure 40. Angiopoietin like protein quantitative results.

Primers were designed for Angiopoietin like protein and 3 replicates from each animal was obtained and normalised against the housekeeper. The interquartile ranges were calculated and the results suggest that the presence of *E.coli* O157:H7 increases the transcription of the gene with a fold change of 1.29 in the challenged group (p=0.04)

The data produced by the qRT-PCR and the microarray does not allow a definitive statement to be made regarding the influence of *E .coli* O157 on the cell cycle as there appears to be contradictory effects when the genes are examined by different methodologies. CyclinC appears to be involved in the transcription of DNA and its presence may indicate increased cycling as may the increased presence of Est-1p protein which mediates TERT access to telomeres in immortalised cells. However Jumonji is a negative regulator in transcription by down regulating pRb phosphorylation and was upregulated as was Zinc-finger 161 in the array assessment. Angiopoietin-1 like protein was also changed but its wide variety of effects on the epithelium makes it difficult to deduce a single effect. The array assessment also does not agree with the direction or magnitude of differentiation by qtRT-

PCR making a final assessment of the overall effect on gene expression, if any, on the epithelium difficult. Nevertheless the FASTA analysis and literature searches revealed that many of the identified genes were involved in pathways that focused on pRb which is phosphorylated as a key control checkpoint of the cell cycle. EHEC strains although not O157 strains have been noted to influence the cell cycle, the effect of colonisation on proliferation was examined.

To determine effects *In vivo* tissue from the same animals used in the microarray and qtRT-PCR were assessed for three markers associated with proliferation and differentiation:
i) Proliferating Cell Nuclear Antigen (PCNA) which is a Cyclin protein that is produced during proliferation and is part of a DNA polymerase complex which allows first strand synthesis in DNA replication, PCNA is normally located within the nucleus of the cells.
ii) Retinoblastoma protein (pRb) which binds E2F transcription factor and restricts it away from its binding site in the promoter region of genes. pRb is normally located within the nucleus of the cells.
iii) Phosphorylated pRb (pRb) which is unable to bind the transcription factor E2F and this allows E2F to attach to its binding site to effect gene expression allowing cell cycle progression.

5.2 Immunohistochemistry for markers of cellular proliferation.

5.2.1 PCNA expression in the crypts of the rectal epithelium.

The cells of the crypt are proliferating constantly throughout the life of the animal. The real time PCR results were suggestive of a change in the rate of this proliferation within the intestine of colonised animals. To test this hypothesis PCNA was selected as a cell cycle marker for immunohistochemical assessment of control and colonised mucosa. The strongest stain was in the nucleus with a minor amount in the cytoplasm. Cells positive for the marker were counted in 5 crypts per section with 3 sections per animal (crypts were determined where both the top and a lower section in contact with submucosa could be seen on a x40 power field). The proportion of positive cells was recorded; analysed using Excel (Microsoft) and interquartile ranges were composed. A diagram representing this is shown below (figure 42. The result suggests that colonisation by *E. coli* O157:H7 is associated with a reduction in this proliferation index within the cattle epithelium (P=0.02). This is presented as Figure 43 with examples of sections produced following staining presented as Figure 41.

Section A **Section B**

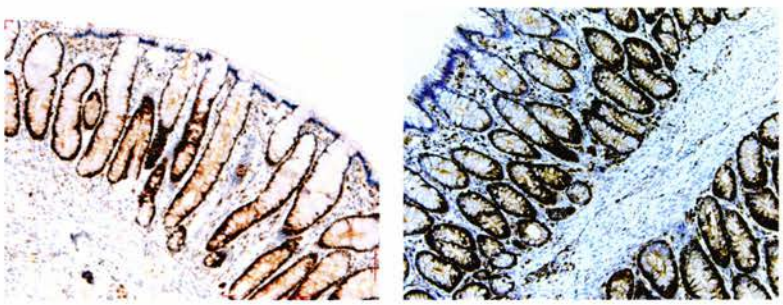


Figure 41. PCNA presence was examined by Immunohistochemistry.
PCNA is an indicator of proliferation and indicated in stained sections by a dark brown colouration. 5 crypts from each animal in the group were examined, and 6 animals were in each group and percentages of epithelial cells positive for PCNA staining were calculated. Section B is a control group while section A is a challenged group, no difference in localisation of PCNA or distribution of cells was noted.

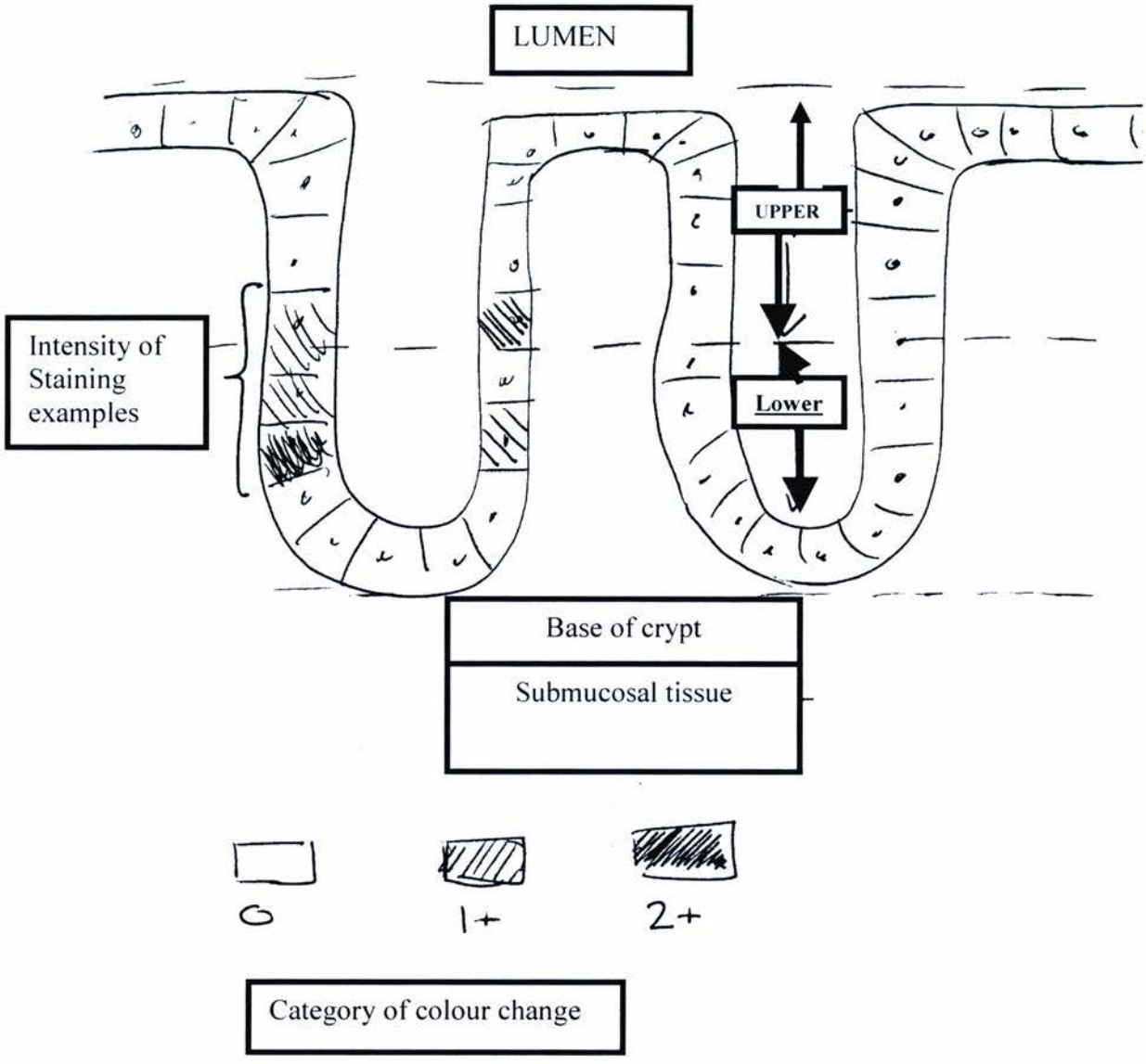


Figure 42. Representation of the crypts counted to assess proliferation.

A full crypt was defined as having a blunt end in contact with the submucosa and a clear shoulder visible at in a 40x power field. The crypt was split at approximately halfway and counts were made of upper and lower portions. The intensity of staining was also measured and graded 0, which had no colour change to 2+ which was strongly coloured

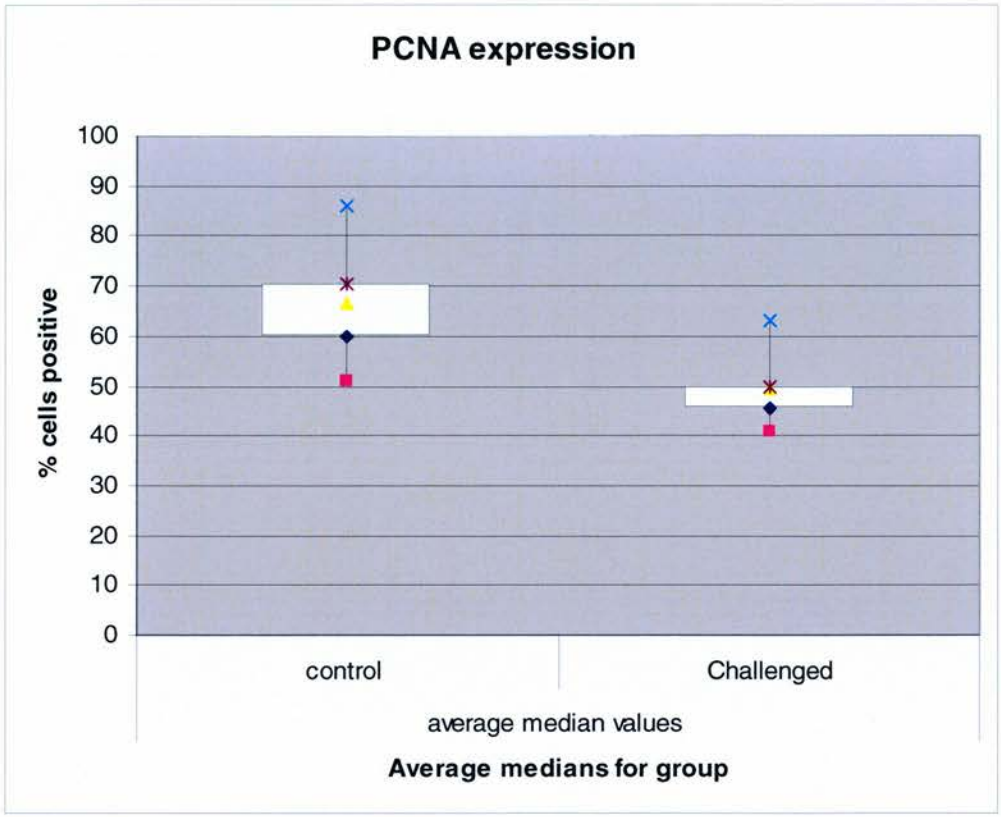


Figure 43. Percentage staining for PCNA.

Cells were stained following probing with an antibody for PCNA a marker for cellular proliferation. 5 crypts per section were counted and the number of positive cells was expressed as a percentage of the total cells present in the crypt and 6 animals in each group was examined and these percentages were analysed using excel. The challenged group have a lower percentage of positively stained cells ($p<0.05$)

5.2.2. Retinoblastoma protein in the epithelium of the rectal mucosa.

pRb localisation and levels were assessed using an IHC assay. Initial visual inspection suggested that the protein was located in two distinct subcellular locations: nuclear and cytoplasmic (Figures 44, 45 and 46) in both control and colonised animals and many cells were positive in both locations suggesting that nucloecytoplasmic shuttling occurred within the epithelial cells. Manual counts of both nuclear-located pRb and pRb located within the cytoplasm were carried out.

In normally replicating epithelium, the majority of pRb would be expected to be expressed within the lower portion of the crypts where most cell proliferation occurs. Indeed there was most evidence for proliferation in the lower part of the crypts in both Control and Colonised animals confirming expectations (Figure 47). However the most intense staining was found in the upper part of the crypt, although the number of cells was relatively small an observation that is not inconsistent with the role of pRb which is also associated with terminal differentiation of cells (fig 48). To quantify staining, the crypts were divided into lower and upper sections and manual counts of the cells positive for nuclear pRb were made (fig 49 and 50).

Section C Section D

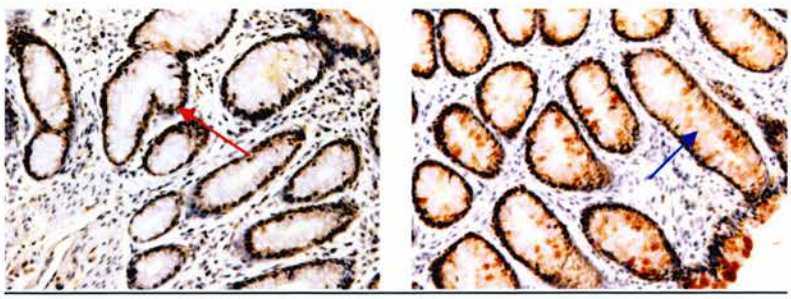


Figure 44. pRb in rectal epithelium.

Colour change was achieved following probing with the appropriate secondary antibody by use of the DAB staining system. Cells positive for pRB were marked with a brown colour (Red arrow) Section C was unchallenged and the majority of staining appeared to be in the nucleus of epithelial cells and the crypts were separated by round lymphoid cells and cells of a stromal nature. The challenged animals (Section B) had more cytoplasmic pRb (Blue arrow) staining suggesting that pRb is located in the cytoplasm compared to the control section.

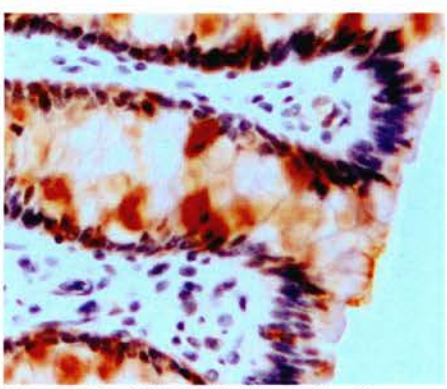


Figure 45. pRb in challenged cells.
High power image of epithelial cells stained for pRb showing staining in the cytoplasm of the cells and limited staining in the nuclei.

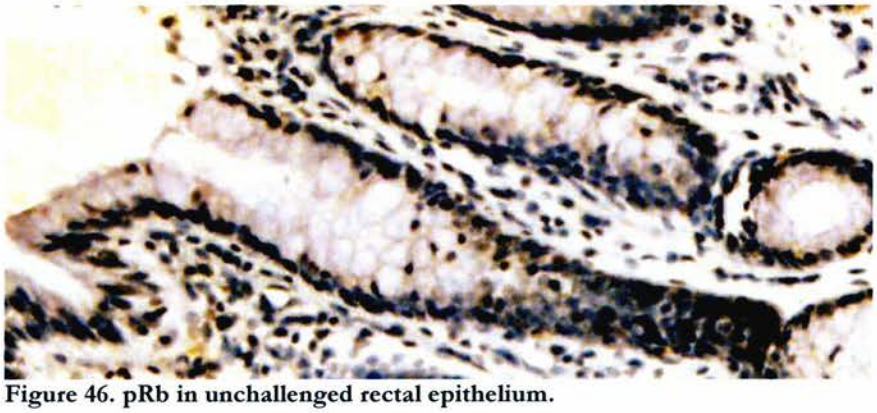


Figure 46. pRb in unchallenged rectal epithelium.

High power image of epithelial cells stained for pRb showing its present in the nucleus of the cells and limited staining in the cytoplasm.

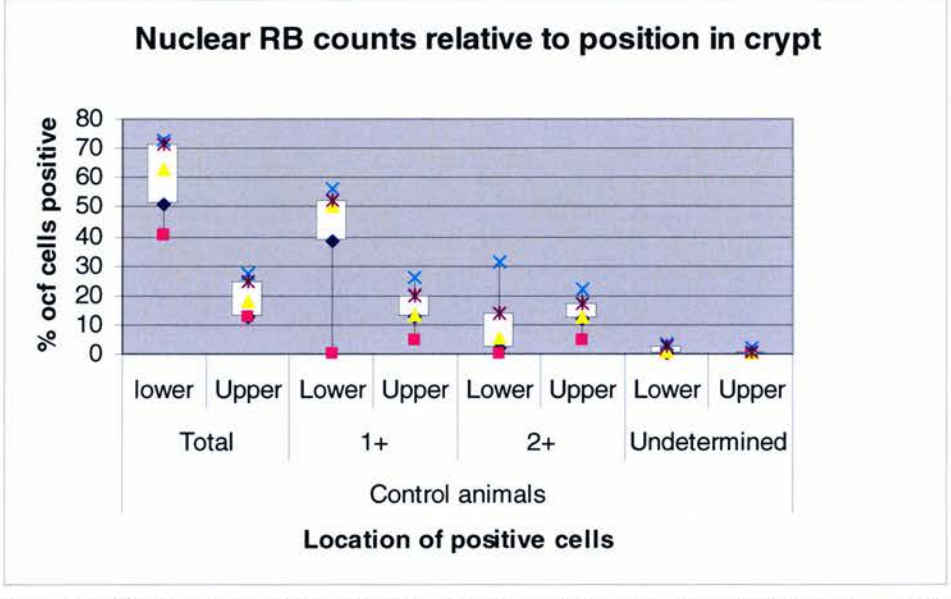


Figure 47. Tissue sections from bovine rectal mucosa were probed with a monoclonal antibody for Retinoblastoma protein.

The resultant colour change was graded into 2 grades 1+ and 2+ depending on the intensity of the colour change. Cells that were unable to be reliably classified were marked as undetermined. Five Crypts were counted and the percentage of cells for each is calculated. The median value for each animal was calculated and interquartile ranges are presented. P-values were calculated between the medians of the upper and lower portions of the crypt. For the total percentage of cells positive the lower portion of the crypt gave higher counts and in the upper portion there is less expression of retinoblastoma protein ($p=0.0001$). The cells categorised as moderately staining showed a similar protein ($p=0.01$). There appeared to be less very strongly coloured cells in the lower portion of the crypt but this was not significant ($p=0.2$). . The number of undetermined cells was not different between groups .and remained in the 3-5% range across the crypts measured ($p=0.1$)

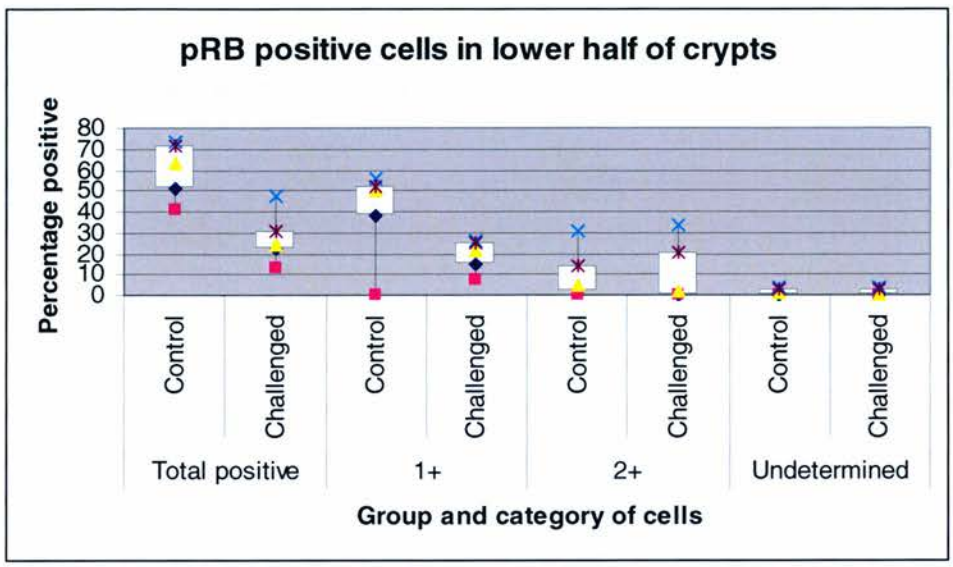


Figure 48. pRb in the lower crypt.
Cells probed for Retinoblastoma protein in the lower portion of crypts were compared between the control and challenged groups for all the categories. For total percentage positive and those cells designated as 1+ there was a significant reduction in the percentage of cells positive for Retinoblastoma in the challenged group.($p<0.001$ and $p<0.05$ respectively) The percentage of strongly marked positive cells was not significant ($p=0.4$). There was no difference between the groups with respect to undetermined cells.

In the lower portion of the crypts there was a lower number of cells positive for pRb in challenged animals (Figure 48) suggesting that there may be a difference in the crypts activity when colonised by EHEC. Observation of the staining pattern of the cells suggested that there were two compartments where pRb staining was observed: the nucleus and in the cytoplasm with challenged animals appearing to have less nuclear pRb (Figure 49). Analysis of this pattern of staining appeared to suggest that the challenged animals have more pRb in the cytoplasm whilst quantisation confirmed that animals that were colonised with EHEC showed more pRb protein in the cytoplasm than those of the control group (Figure 50).

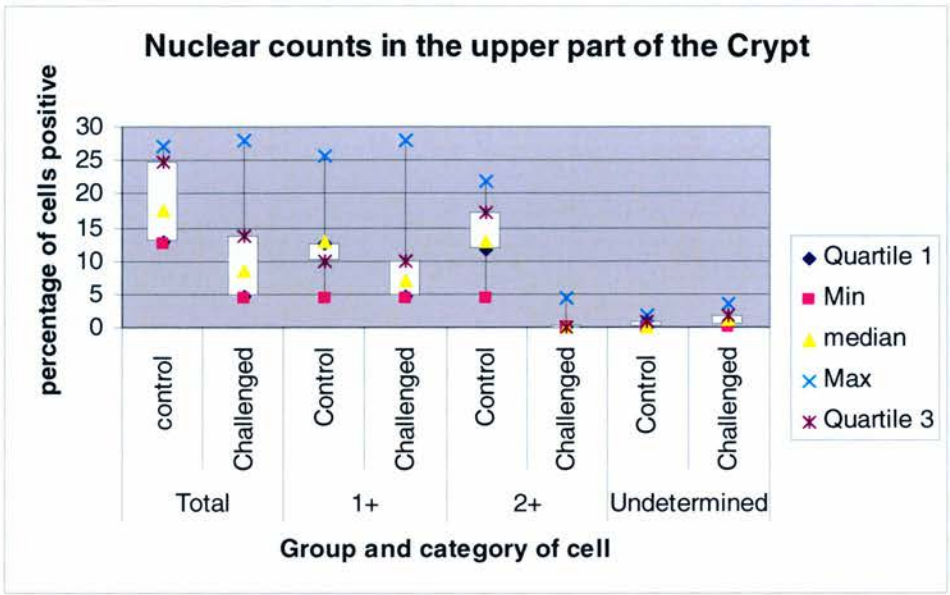


Figure 49. pRb in the upper crypt.
Rectal mucosa was probed with a monoclonal antibody and a Dab colour change was used to identify cells and categorise them with respect to the intensity of the colour change. For the upper part of the crypts there was no difference in total cells marked as positive (p=0.07). However the difference between the very strongly stained cells is greater in the control group probably representing the reduction of terminal differentiation in the challenged animals (p=0.001). The undetermined cells were not different between the two groups (p=0.1)

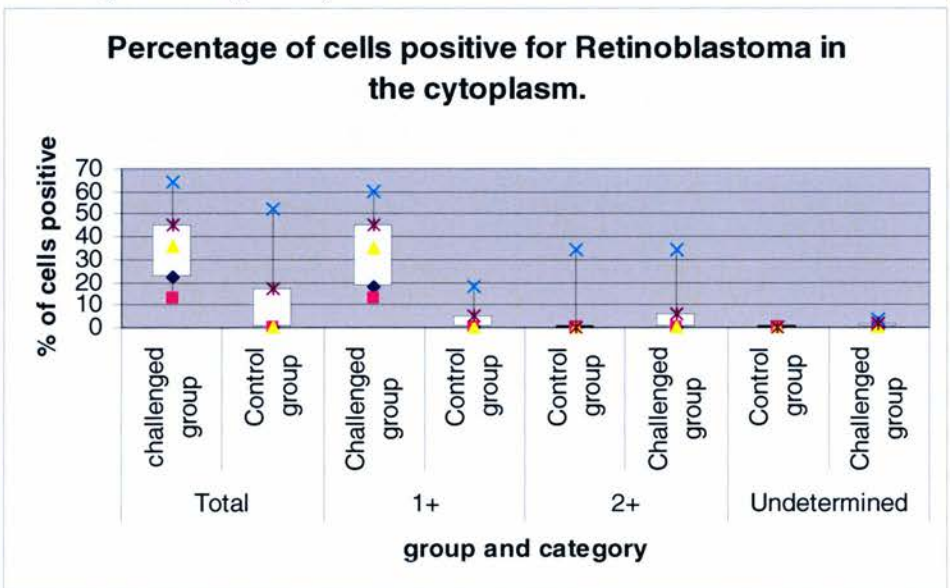


Figure 50. pRb in the cytoplasm.
Colour changes associated with retinoblastoma expression was noted in the cytoplasm of the epithelial cells lining the crypt. Cells were counted into groups depending on colour change intensity or undetermined due to location or shape. More cells in the challenged groups were positive for retinoblastoma in the lower part of the crypt (Total, $p<0.05$ and 1+ $p=0.005$). However very strongly staining cells were (2+) ($p=0.09$) were not significantly different.

5.2.3 ImageJ Analysis of images for Rb distribution.

In an attempt to account for operator bias in the counting of the cells an automated system was employed. Using Adobe Photoshop the images obtained using an Olympus microscope and camera were converted to black and white. ImageJ software was used to measure the area covered by black representing staining. However although this supports our hypothesis that more pRB is in the cytoplasmic compartment (Figure 51). It is likely that this method measures other structures hence the areas measured are unlikely to be an accurate reflection of the area covered by pRb. There is reduced chance of operator bias, but not excluded as section selection is still done by human eye.

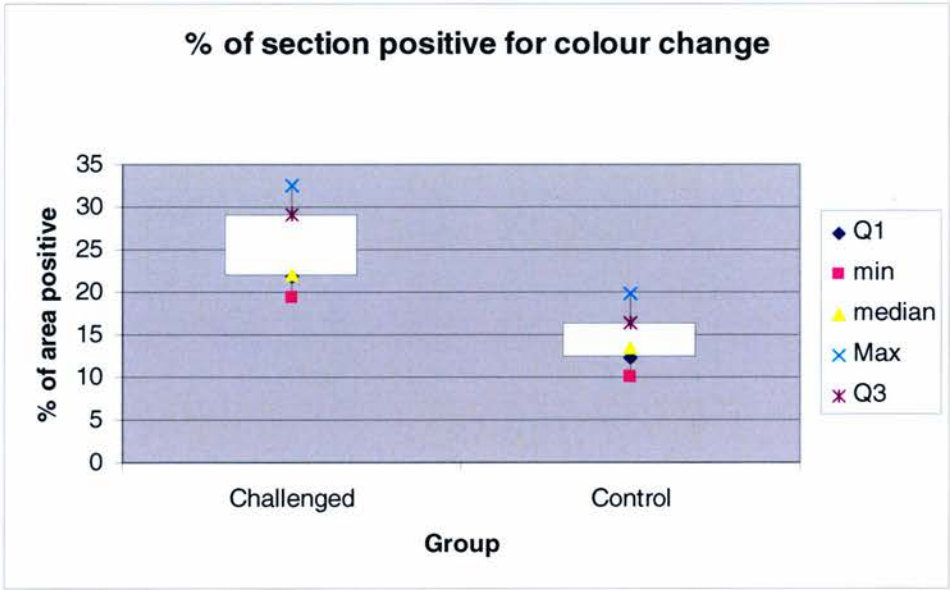


Figure 51. ImageJ analysis of pRb.
3 sections stained as before were photographed using an Olympus microscope and Olympus D70 camera. The digital images were opened in Photoshop and the colour change was delineated using this package and masked with black. The file is accessed with ImageJ software (NIH) and the total area covered by the threshold pixel strength. The medians for each animal were obtained and are presented in each group in the graph above. There is an increase in the area with the appropriate colour change in the challenged animals supporting the cell counts in the cytoplasmic compartment. ($p>0.05$)

5.2.4 Phosphorylated Rb expression

The expression of phosphorylated Rb was also examined using an antibody to pRb phosphorylated at serine residue 780. Phosphorylation at this site allows release of the E2F proteins to function as promoters for transcription factors required for cell replication. There was no difference between the control and challenged groups for proportion of cells expressing phosphorylated pRb (Figure 52). In addition the subcellular distribution of the protein appeared to be similar between groups (Figure 53).

Section E **Section F**

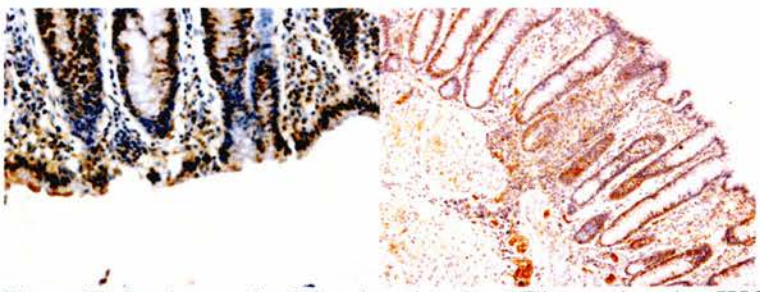


Figure 52. Sections stained for phosphorylated Rb protein using IHC.
This protein appeared to be located in the nucleus of epithelial cells of the rectal epithelium of both the colonised sections (Section E) and the control (unchallenged) sections (F). Visually there appeared to be no difference between sections for distribution of this protein

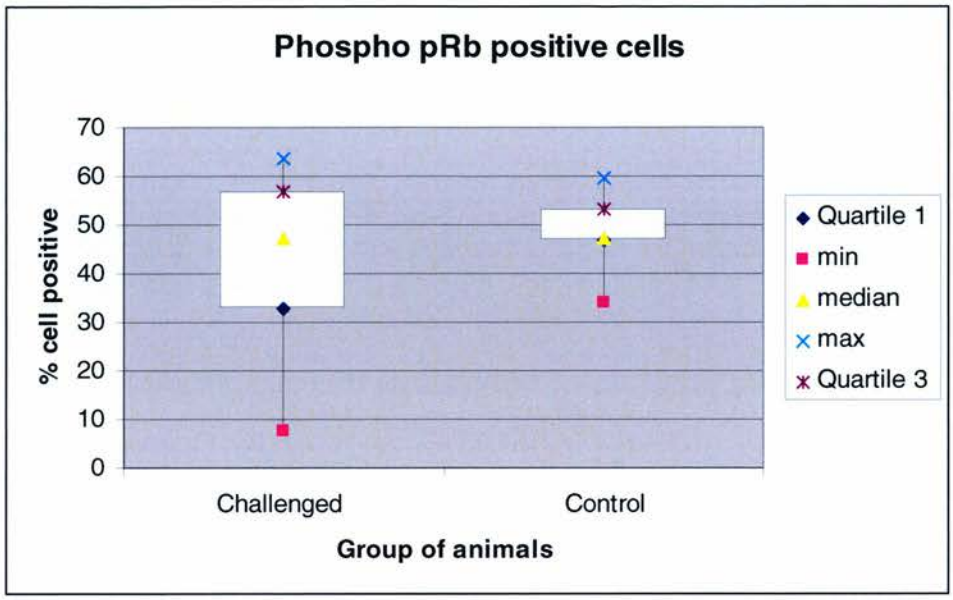


Figure 53. Phospho-pRb staining in rectal epithelium.
Cells were stained in the same manner as before. However an antibody for pRb phosphorylated at the serine 780 residue was used as a primary antibody. This residue is phosphorylated at a later stage than the other residues and represents pRb that is unable to restrict the access of E2F to the binding sites on the DNA. There is no difference in the groups for number of cells containing phosphorylated pRb this suggest that EHEC is not inhibiting the phosphorylation of pRb. Observation of the cells revealed a purely nuclear localisation of the phosphorylated pRb in comparison to the more widely distributed pRb ($p>0.05$)

5.3 Discussion.

EHEC is an important group of human pathogens, causing diarrhoea and other more severe symptoms, including haemorrhagic colitis and haemolytic ureamic syndrome. A very small percentage of patients will suffer fatal disease. Infection is caused by the contamination of the food chain with faecal matter derived typically from bovine intestinal contents. Poor slaughterhouse technique or poor handling of bovine faeces on farm allows food chain and environmental contamination and poor hygiene and cooking procedures allows ingestion of viable bacteria. The very small infectious dose required to initiate an infection in human cases makes removal of the bacterium from the food chain very difficult. EHEC colonises a very specific part of the intestine, the recto-anal junction (Naylor et al., 2003; Dean-Nystrom et al., 1999). Like many bacteria it is believed that this bacterium has adapted to colonise a specific niche and the pathogenicity factors are an aid in this process. The ability to interfere with this colonisation would prevent the bacterium from contaminating the food chain thus reducing the human health implications

To colonise the host, bacteria may influence the environment in which they find themselves by manipulating immune responses along with other physiological processes (Tesh, 1998; Kaper et al., 2004). This allows the bacteria such as *E. coli* to manipulate the host into offering a more favourable environment for survival. The intestinal epithelium where EHEC persists offers advantages and challenges to bacterial survival. EHEC remain extracellular and in the course of colonisation of cattle do not normally enter tissues. This affords it a degree of protection from the humoral immune system. Adaptive immunity does not appear to significantly affect colonisation and there is little evidence to support a pathogenic response in cattle that would be associated with inflammation (Baehler and Moxley, 2000) (Mahajan PhD thesis Edinburgh University 2006). This observation that there

appears to be little pathological change in cattle is unexpected as *E. coli* O157:H7 does elicit pro-inflammatory factors from bovine epithelial cells (Chapter 3) and cellular changes do occur in colonised cattle. These changes include the attaching and effacing lesions and cytoskeleton rearrangement in the colonised epithelial cells although there is no notable inflammation. This lack of an inflammatory response argues against a profound pathogenicity for the bacterium. These observations are not consistent with a purely commensal relationship as this has been understood until recently, where virulence factors explain the differences between mucosal responses to commensal and pathogenic bacteria (Kelly et al., 2005). The bacteria in the intestine are however subject to control of the innate immune system which functions to control pathogens before the humoral immune system initiates a response and may be involved in control of the commensal bacterial load of the intestine. Innate immunity also incorporates those aspects of the normal physiology that would make it more difficult for a pathogenic bacterium to colonise the intestine such as mucus production from goblet cells and the ability of certain bacterial including StcE of *E. coli* O157:H7 (Grys et al., 2005) species to degrade; this may be important in pathogenesis (Mantle and Rombough, 1993). Further investigation is required to investigate the manner in which *E. coli* O157:H7 can influence the the epithelium to allow colonisation. In order to understand this interaction the epithelial cells an assessment of global transcript production was attempted by isolating the cells of interest from the intestine and extracting the mRNA produced by the cells.

Analysis of transcript production by epithelium colonised by EHEC when compared to control epithelium revealed a limited subset of transcripts that had altered expression levels. In line with common practice those that had a 2 fold or greater change in expression either as an increase or decrease were considered to be of sufficient magnitude that indicated

there might be a true change occurring. BLAST analysis of these transcripts allowed the identification of the genes represented by the EST sequences and biologically relevant groupings could be postulated. Most of these genes had a function in cellular proliferation when the literature was consulted and a simple diagram of possible relationships and roles is presented (Figure 54). Some were integral proteins in the cell cycle, for example CyclinC which allows exit from the Go phase of cell cycle, Est-1p like protein is involved in the maintenance of telomeres in cells. Jumonji domain containing protein 2B has been demonstrated to inhibit cell proliferation through the down regulation of CyclinD1. The remaining two proteins have a more ambiguous role in cell proliferation, with ZFP161 interacting with other proteins including Zinc finger 295 and c-myc to give bi-directional control of transcription. Angiopoietin-1 like protein has been demonstrated to have a multiplicity of roles including a mitogenic effect on blood vessel endothelium. qtRTPCR assay of the selected group of genes did not always conform to the magnitude or the direction of the changes as demonstrated by the microarray assay; this along with the multitude of effects associated with some of the selected proteins made identification of an associated phenotype very difficult. The data provided by both sets of experiments is contradictory and requires further validation to determine if any of the proteins examined are in fact influencing the epithelium of the cells colonised by *E. coli* O157:H7.

The use of housekeeper genes is somewhat controversial but required for ensuring and correcting for technical bias in the experimental set up. As amplified cDNA from the array experiment was used in the real time validation it was considered appropriate to look at the array data for a gene expressed at similar levels to the genes of interest and not apparently altered by the experimental set up. Any difference between the challenged group and the control group with respect to the housekeeper could therefore be accounted for.

This approach is outlined in the literature (Szabo et al., 2004). It is noted that it normalisation could alter the interpretation and produce false positives. To adjust for this the use of multiple genes for normalisation may give a more accurate assessment of basal transcription in the rectal epithelium.

Literature search identified that a common effect of these proteins was an influence on the cell cycle. All of these proteins appeared to influence retinoblastoma protein a key protein in cell cycle control and if there was an influence on the cell cycle it is likely that this protein may be affected. However as it is so key to the regulation of cellular proliferation it is possible that other influences are more important than the proteins chosen in this study. Imunnohistochemistry was chosen to examine pRb and PCNA in an effort to determine if there was an influence on the epithelium following colonisation.

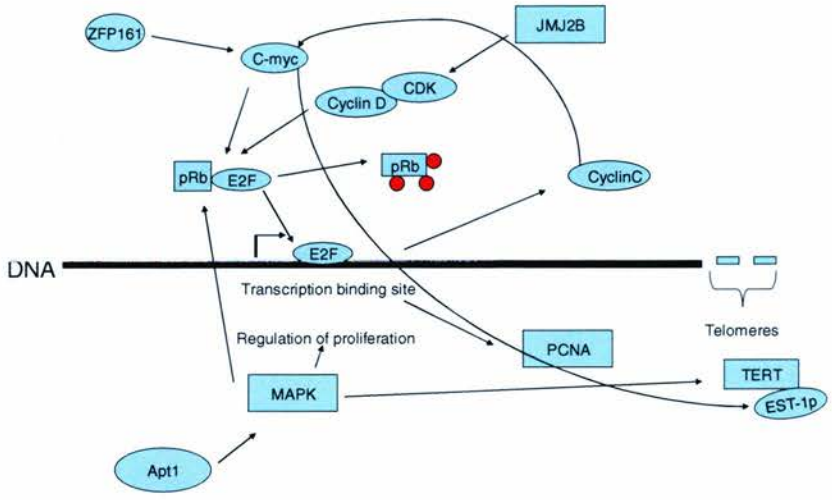


Figure 54. Schematic of genes identified.
A simplified diagram of the interactions between the genes identified by microarray and further analysed by qtRTPCR, as well as those examined by IHC. Arrows indicate an interaction but in most cases the effect is complicated by other interactions so a determining an effect from literature is complicated. Phosphorylation of pRb is indicated by red circles and this event is a regulatory checkpoint for cellular proliferation

5.3.5 PCNA is down regulated within the epithelium of colonised cattle

PCNA is a stimulating factor for DNA polymerase δ; it interacts with this protein to allow DNA replication and has been used widely to indicate proliferation within tissues. Using routine IHC and manual counting of cells that were positive for PCNA staining within the crypt it was found that there were significantly fewer positive cells in challenged cattle. This suggests that the colonised cattle have a lower proliferative rate within the crypts and combined with the results of the qtPCR techniques it is suggestive that EHEC is able to influence the cells of the crypt to slow proliferative rates and preserve the niche that EHEC has evolved to colonise.

5.3.6 Retinoblastoma protein is sequestered away from the nucleus in colonised cattle

Retinoblastoma protein is a key "gatekeeper" protein used by the cell as one of the controlling signals that allows the cell to replicate. The hypophosphorylated form binds E2F transcription factors into a complex with HDAC (Weinberg, 1990) and is normally retained within the nucleus by a bipartite nuclear signal (Zacksenhaus et al., 1993; Verona et al., 1997). Phosphorylation of pRb (by Cdk3, for instance) releases the E2F factors allowing shuttling to the nucleus where binding to the appropriate transcription site can occur; the proteins that are required for cell proliferation including for example replication protein A (Polager et al., 2002) are then transcribed (Ikeda et al., 1996). In the intestine the crypt is the primary site of proliferation for the epithelial cells, cells that are constantly replicating and migrating up the villi where they replace the senescent cells of the tips. As the major site of replication is the crypt pRb expression would be expected to be increased in this area and in fact this was observed in both control and colonised animals. The upper part of the crypt and epithelial cells at the surface showed very little unphosphorylated pRb expression consistent with an area of limited cellular proliferation and a majority of terminally differentiated cells in the colonised animals. However some pRb was still present in the upper crypt and this may reflect some terminal differentiation of lymphocytes and goblet cells occurring in the epithelium. However in the challenged animals there was a significantly greater presence of pRb suggesting that the protein is retained in the cytoplasm.

The percentage of cells positive for phosphorylated pRb is approximately the same within each group, Phosphorylated pRb is unable to bind E2F factors and these are then

able to activate transcription factors leading to transcription of genes required for cell cycle progression. Hypophosphorylated pRb is able to sequester the transcription factors and inhibit the transcription of cell cycle factors. It has been proposed that it is the balance between the two states that is the critical event that pushes the cell into proliferation. Once the correct ratio of Phosphorylated pRb is reached and enough transcription factors are released then cell proliferation occurs. The retinoblastoma protein in the control animals is located within the nucleus of the lower crypts with only a minor percentage of cells expressing pRb in the cytoplasm. In the challenged animals the subcellular location of pRb changes from its normal nuclear position with much more being expressed within the cytoplasm; this cytoplasmic pRb may be able to sequester E2F in the cytoplasm away from its site of action as it has been demonstrated as occurring in some cell lines (Jiao et al., 2006), and may present a way for proliferation to be controlled. The presence of pRb in the cytoplasm in the upper parts of the villi suggests a prolonged or sustained sequestration in the cytoplasm which may also retain E2F away from the binding site in the transcription activation site of genes. The approach here utilises an IHC approach and the colour change associated with DAB staining. The resolution is not as high as other techniques and while it is tempting to conclude that pRb is being held in the cytoplasm the data is not conclusive; it may be prone to operator bias but attempts to automated the process using ImageJ software while not without its problems supports the conclusion that pRb is retained in the cytoplasm. Fluorescence or confocal imaging would improve resolution and allow definitive conclusions to be made about the location of the pRb within the epithelial cells.

5.4 Summary.

The intestine is constantly being challenged by pathogens and the harsh environment of the lumen. Cells are constantly damaged and require removal and replacement. This is a carefully controlled process that balances the replication rate of cells in the crypts and the loss of these cells from the villi tips. Too little replication or to great destruction of the cells, as seen in some bacterial infections e.g. *Salmonella*, and the villi will shrink in size and absorptive area will be lost. Conversely too great a rate of replication and uncontrolled differentiation will result in the formation of large masses of cells at the intestinal epithelium. This is frequently caused by oncogenic mutations in the cell but other insults (such as chronic inflammatory responses) and those viral infections involving oncogenic viruses may also be involved. Bacteria that promote epithelial proliferation include *Citrobacter rodentium* (another attaching-effacing pathogen), *Helicobacter pylori* and *Lawsonia intracellularis.*

The cell cycle is divided into discrete steps, termed G1, G2 S and M phases, with G0 usually referred to as a quiescent cell, although the cells in G0 may be undertaking their mature functions and are terminally differentiated. A simplified scheme is shown below (Figure 55). Phosphorylation of pRb is required for cells to pass from G1 to the S-phase and this results in the release of the E2F factor that transcribe the DNA replication machinery required for the DNA duplication that occurs at this stage.

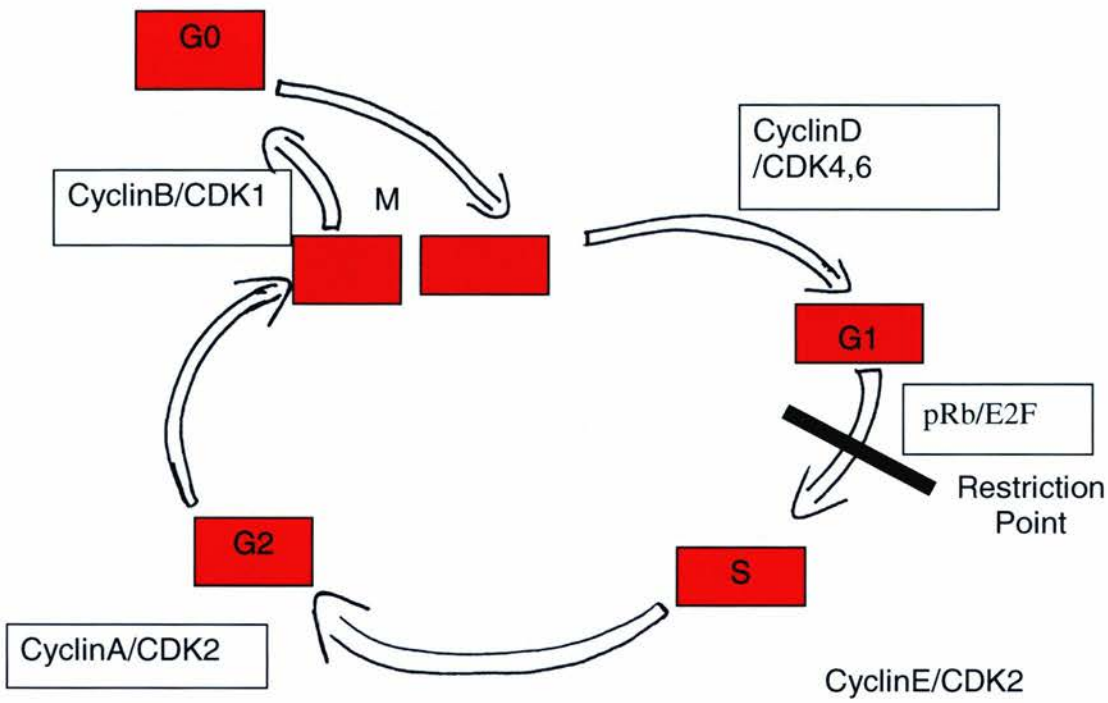


Figure 55. A simplified description of the cell cycle.
The quiescent (nonreplicating) cell can enter the cycle under the influence of mitogens where DNA replication cell growth and division can start. Each of these processes is controlled by the presence of Cyclins and their paired Cyclin dependent kinases. The restriction point is where pRb is able to exert its effect and phosphorylation of this protein commits the cell to division.

The G1 phase contains within it a critical point where signals are integrated to create a decision about further progress through the cell cycle. The cell can progress, pause or leave the cycle dependent on the signals it has received. This point is termed the Restriction point in the Eukaryotic cell. The Restriction point is passed when the ratio of signals mediating the phosphorylation of Rb are in excess of those mediating the hypophosphorylated state. The phosphorylation of this protein allows the release of the E2F factors and these can then perform their function as transcription factors. A great many cell pathways focus on this single event and there would appear to be a critical level of phosphorylation that allows the cell cycle to proceed.

Analysis of gene expression in EHEC colonisation revealed changes in a number of genes that may be involved in the proliferation of cells within the epithelium. These included Zinc finger protein 161, Est-1p like protein, Jumonji domain containing protein 2B, Cyclin C, and Angiopoietin like protein 1. The correlation between qtRT-PCR and microarray output was inconsistent and therefore it is difficult to determine if there *E.coli* O157:H7 has an influence on these proteins.

EHEC encodes a large number of virulence factors that are important in the pathogenesis of human disease and some of these factors, CIF, CDT and CNF are able to induce cell cycle block at G1/S transition. Examination of the epithelium for the presence of proliferation indices was used in an attempt to clarify the effect on the epithelium and an effect on proliferation was noted in animals colonised by *E. coli* O157:H7, with epithelium appearing to have a lowered proliferation rate. However Walla Walla 3 the O157 strain tested here does not carry factors that have been associated with cell cycle arrest therefore requiring another mechanism by which this inhibition is mediated.

Chapter 6

General Discussion

General discussion

E. coli O157:H7 (EHEC) is an important zoonotic pathogen that causes diarrhoea and has been implicated in HUS and HC. These syndromes can cause fatalities in patients. It is found in the faeces of cattle as these animals are important reservoir hosts. Contamination of the food chain is important in the epidemiology of the disease. The pathogenesis of this disease has received much attention over the last years.

It is known that shigatoxin (Stx) is a major virulence factor in the pathogenesis of EHEC associated disease. Intoxication by Stx blocks protein elongation at the ribosomes and this leads to cell apoptosis which is followed by loss of epithelial integrity. Fluid loss through the intestinal epithelium and the renal system results in dehydration and possibly serious sequalae including chronic renal failure and death.

In patients affected by EHEC associated disease clinical signs are associated with virulence factors carried by EHEC. These include flagella and LEE encoded factors; of which flagella appears to predominate in the pro inflammatory responses to colonisation. Its interaction with TLR-5 induces transcription of Il-8 and the expression of the protein will promote chemotaxis of neutrophils to the site of colonisation. The presence of these neutrophils will further promote damage to the epithelium and worsen the already impaired epithelium following Stx intoxication. The LEE encoded factors including TIR and intimin induce A/E lesion formation on epithelial cells in the intestine and this may reduce the absorption of fluids from the intestine contributing to the diarrhoea noted in clinical cases.

In the bovine no clinically apparent inflammatory response is noted so an EHEC strain unable to express flagella was used to challenge the model system. IL-8 transcript production was reduced when challenged by flagellin negative EHEC, however transcript reduction was incomplete and other factors were still causing pro inflammatory responses in

the epithelium. Intestinal resident *E. coli* bacterium representing both motile, (expressing flagella), and non-motile bacteria were examined and neither was demonstrated as being able to increase Il-8 transcription.

This suggests that a combination of factors are required to cause Il-8 transcription and since the ability to cause attaching and effacing lesions is an important part of EHEC pathology, the presence of the LEE pathogenicity island was examined to determine its ability to elicit Il-8 transcription. It was not possible to determine a difference between the strains tested. The attaching and effacing lesions caused by LEE encoded proteins may affect the tight junctions around the cell; in humans it may increase the permeability of the epithelium allowing flagellin to access the TLR-5 receptor which in undamaged epithelium is retained to the basolateral aspect of cells (Figure 56). This may partially explain why EHEC is not causing inflammation in the bovine host although EHEC expresses flagella and in the model system induces IL-8 transcription; therefore is flagella is likely to be pro inflammatory in the bovine but flagella may retained in the lumen away from the TLR5 receptor. This observation and the differentiation of tight junctions between human and bovine epithelium may be key to the difference in responses between these two species. However as noted the position of TLR-5 and the state of the polarisation of this model system is as yet unknown and any conclusions about the data presented here must be drawn with care.

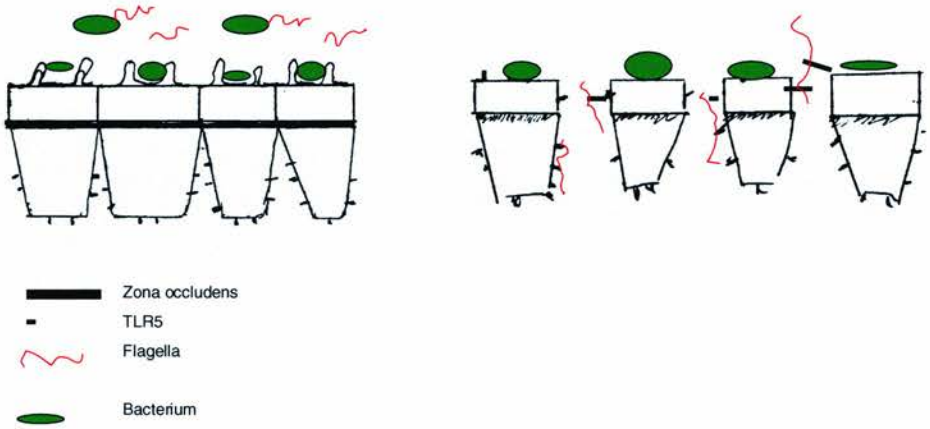


Figure 56.A model of Tight junction disruption and consequences for inflammation.
In the bovine (Left) the zona occludens remains intact which separates the apical and basolateral compartments and therefore TLR-5/Flagella interation is limited. In the human (Right) EHEC infection disrupts the Zona Occludens exposing TLR-5 and allowing interaction with Flagella as TLR5 leading to an inflammatory response. As the bacterium can cause A/E lesions in the bovine other mechanisms are likely to be ameliorating the bovine response

Little is known about the possible mechanisms and pathways that are involved in the interaction of EHEC with the normal bovine epithelium. The pro inflammatory ligands and particularly flagella and LEE pathogenicity island associated proteins are able to induce inflammatory associated transcripts such as those for IL-8 (Chapter 3) in bovine epithelial cells. Comparison with commensal bacteria, rather than residents, suggests a difference between these resident strains of *E. coli* and *E. coli* O157:H7 as flagella expressed by commensal bacteria is not able to induce pro inflammatory transcripts. This suggests that there is a continuum of bacterial pathotypes in the intestine from benign to fully pathogenic and EHEC may be influencing the epithelium in unsuspected ways. However to confirm the model suggested, further work is required. It would be interesting to utilise Ussing chambers to measure changes in trans-epithelial resistance that may occur following colonisation with bacteria exhibiting defined mutations relating to flagella, including mutants that fail to express flagella, show no motility despite expressing flagella or fail to demonstrate

chemotaxis. This would allow changes in the epithelial tight junctions induced by EHEC to be assessed and compared to commensal bacteria with and without flagella. As it is postulated that tight junction proteins will change distribution following colonisation by these bacteria immunohistochemical examination of associated proteins such as ZO-1, occludin and others may prove productive in demonstrating changes in these proteins.

As there is a paucity of information about the interaction of *E.coli* O157:H7 with the bovine host in terms of host molecular changes microarray was utilised to examine the responses by bovine epithelial cells as it can interrogate potentially thousands of genes. When combined with Laser capture microdissection used to isolate a single cell type it is a powerful technique and can demonstrate numerous pathways that may be altered by pathogen interaction. This is a novel approach to answering the question of how a bacterium interacts with the host and demonstrates the power of microarray when combined with the correct sample selection.

Samples of the recto-anal junction where colonisation is thought to primarily occur (Naylor et al., 2003) were removed from calves following post-mortem and frozen prior to LCM and microarray analysis; using this technique a small subset of genes were identified that were differentially regulated following EHEC colonisation of weaned calves. These genes were identified and grouped following appraisal of the available literature into biologically relevant groupings of which the largest appeared to be involved in the cell cycle and control of cell proliferation. These genes included CyclinC, Est 1p like protein, Jumonji domain containing protein 2B, Zinc finger 161 and Angiopoietin-1 like protein. However these data were inconsistent with the Qt-RTPCR, techniques used to validate the array. It is therefore impossible to conclude that EHEC is influencing the expression of these genes. It s empting to speculate that these proteins may be responsible for the phenotype discussed in

chapter 5 and further discussed below but until further work is carried out to validate the results outlined here it remains the role of these proteins are speculative. To validate the results and resolve this confusion further investigation is required. IHC examination of the proteins either in situ or as extracted proteins (Western blot) would allow determination of the expressed levels of each gene in the cells of interest.

As stated above the data from the qtRT-PCR and array experiments was contradictory and failed to provide support for an influence on the epithelium however the hypothesis that there may be an influence on the epithelium remained tantalising; in order to determine whether EHEC colonisation is able to influence the rate of proliferation Immunohistochemical techniques to detect cells expressing Retinoblastoma and PCNA an antigen that is expressed in replicating cells were used. EHEC colonised cattle displayed a lower proportion of replicating cells than in the uncolonised animals suggesting that EHEC influences the proliferation rate in the epithelium. As EHEC is intimately attached to the cells of the epithelium it is possible that the replication rate is being lowered by the bacterium as a strategy to allow a prolonged period of colonisation by limiting cells that are lost from the tips of the villi. This represents the first time that evidence for a direct influence on the proliferation rate has been shown, although it has been suspected that this may occur from indirect evidence (Magnuson et al., 2000) and *in vitro* work (Marches et al., 2003).

Control of cell cycle is complex , the cell cycle has a regulatory check point that is controlled by the retinoblastoma protein (pRb) which is phosphorylated to release E2F, a transcription factor, and this allows cell replication to occur (Ikeda et al., 1996). pRb was implicated in pathways leading to and from the genes identified by the microarray analysis for example Jumonji domain containing protein 2B down regulates CyclinD which along

with its associated kinase and thus limits pRb phosphorylation and cellular proliferation (Ohno et al., 2004). JMJ2B function has only been demonstrated as being active in the development of cardiocytes and has not been implicated as being altered by bacterium and as such may represent a novel target for bacterial protein.

The other proteins that were indicated in the microarray are also implicated in the cell cycle, CyclinC is part of the DNA polymerase complex and its presence would indicate an increase in proliferation; this is contraindicated by the PCNA data and the accumulation and CyclinC may have other roles in the cell cycle that have yet to be demonstrated (Johnson and Walker, 1999). Similarly Angiopoietin-1 like protein has a multitude of roles and the full role in EHEC colonisation and cell cycle have yet to be confirmed (Arai et al., 2004; Dallabrida et al., 2005). Est-1p like protein is associated with telomerase function which acts to maintain telomeres and over-expression of the telomerase enzyme has been associated with cell immortalisation (Taggart and Zakian, 2003). The analysis of the array did not definitively suggest that proliferation was up or down regulated by EHEC colonisation due to the poor correlation between this data and Qt-RTPCR techniques. Despite this it is hypothesised from the IHC data that EHEC is slowing proliferation by epithelial cells in the intestine as a strategy to extend its colonisation of its niche at the recto-anal junction.

As the proteins implicated by the array appeared to have been involved in cell cycle regulation and a key regulator is pRb this and the phosphorylated form of pRb were examined by IHC; there was an alteration in the cellular location of pRb from the nucleus where it has its site of action to the cytoplasm (chapter 5). The percentage of nuclear pRb was also lower in challenged animals indicating lower proliferation and these may be part of the mechanisms by which EHEC can alter the rate of proliferation. It has not been shown

that bacteria can alter the subcellular location of pRb prior to this and changes in the location of this protein may represent a novel strategy for bacterial control of the cell cycle.

The mechanism by which EHEC influences this alteration in proliferation is unclear but the implication of CyclinD raises some intriguing possibilities. As a characteristic feature of EHEC colonisation A/E lesions occur in the epithelium and there is alteration of tight junctions associated with these changes. Tight junction proteins are linked to the control of epithelial cell proliferation and orientation (Sourisseau et al., 2006b; Zahraoui, 2004; Balda et al., 2003a) and examination of the location of some of the proteins such as ZONAB may indicate if they are relevant. Cell to cell adhesion provides critical control of epithelial cell growth and both anchorage dependence and contact inhibition of cellular growth influence the growth of cells in the epithelium. These signals have roles in controlling both the rate of epithelial proliferation and the polarisation of cells. Once the restriction point is passed however the cell then becomes unresponsive to cell contact and proliferation is reduced. Cell to cell adhesion is mediated through two related structures, the tight junction and the desmosomes. Cell to extracellular matrix adherence is mediated through the hemi-desmosomes, though similar proteins are likely to be found in all. The interaction of these with the extracellular environment is likely to be involved in transducing a proliferative signal and allowing the cells to pass through the restriction point.

The tight junction is a polarised structure and in the mature cell functions to decrease paracellular permeability. Located near the apical surface it consists of a number of proteins including the claudin family, occludins, the Jam family of membrane proteins and the ZO family of junctional adaptors. These mediate attachment, paracellular permeability and polarise the lipids of the cell membrane particularly the outer leaflet (Matter et al., 2005b). The cell density has been shown to suppress proliferation of epithelial cells(Frankel

et al., 2005) through increased density of tight junctions and their associated proteins. Proteins in the tight junction have been extensively studied in respect to their ability to manipulate the cell cycle. The best characterised is ZO-1, an adaptor protein of the TJ complex. This protein interacts with a host of proteins including F-actin, the occludins and ZONAB. Of these ZONAB (ZO-1 nucleic acid binding protein) has been shown to have a role in the proliferation of epithelial cells (Sourisseau et al., 2006a). This protein is a Y-box transcription factor and is sequestered to the tight junction by interaction with a SH-3 domain located within ZO-1 thus preventing access to the nucleus by this protein (Balda et al., 2003b). ZONAB also binds CD4 a DNA binding kinase that activates Rb. ZONAB function is guided by the interaction with RalA. These RalA/ZONAB complexes have been shown to increase in increase in cell dense epithelial cell culture models. This alters the activation state of the protein as well as sequestering it into the cytoplasm. CDK4 a cyclin dependent kinase is down regulated through the actions of ZONAB. Additionally the down regulation of Cyclin D1 is brought about by the cytosolic sequestration of ZONAB. This was thought to be by the down regulation of CyclinD mRNA expression following this sequestration; however the translation of cell to protein can be inhibited by protein kinase C signalling. E-cadherin (another tight junction protein) can inhibit this pathway and promote cell survival when the balance of signals favours apoptosis (Tunggal et al., 2005; Le et al., 2002; Day et al., 1999). The down regulation of cyclin D1 by PKC is through the inhibition of Cap dependant initiation and this occurs by the activation of a suppressor protein which binds with a cap binding protein (Zyrek et al., 2007b). EHEC is able to influence the tight junctions and the modification of the junctions may lead to alteration of the protein sequestration in unexpected directions. A/E lesions contain actin and other cell membrane proteins and may also bind factors like ZONAB with similar inhibition of proliferation. The

presence of A/E lesions may simulate the increasing cell density seen in ZONAB regulated proliferation and induce the change of proliferation seen within the epithelium of colonised epithelium.

To properly examine this over time would require the identification or preparation of a model system with appropriate characteristics to allow the study of colonised tissue over the extended period required to generate this effect, estimated to be about 10 days. Immortalised tissues are not suitable as they have escaped normal cell cycle checkpoints and so may not respond to the colonisation by EHEC in an analogous manner. Human epithelial cell derived lines are likely to interact with EHEC with a variety of proinflammatory mechanisms which may obliterate the response expected by our hypothesis therefore a cattle derived model system would be preferred. *In vitro* organ systems utilising harvested tissue from abattoir or other sources are a possibility and systems have been demonstrated that can maintain these for up to 14 days. *In vivo* gut loop systems also provide further possibilities for examining host pathogen interactions and providing material for further investigation. There are model systems that can provide for prolonged culture times but these have so far been only used for less than a day (Aich et al., 2007; Menge et al., 2004). It may be possible to extend this time period by modifying the surgical technique to bypass gut sections and extend the viability of the tissue. With this material further areas of investigation would include examination of pRb localisation and its retention in the cytoplasm over time as the cyclin dependent kinases have a temporal role and may alter the site of this protein (Jiao et al., 2006). The phosphorylation state of many of the kinases may also be of interest as the activation of these proteins relies on phosphorylation. The effects on tight junction in the gut loop model systems and correlation of proliferation with colonisation may also provide useful insights into EHEC pathogenicity. Although A/E lesions are suggested as the

mechanism by which *E. coli* O157:H7 causes this restricted proliferation there may be specific virulence factors required to produce this phenotype. *E. coli* O157:H7 does not carry any of the factors that have been implicated in cell cycle block (CIF, CDT) and that are carried by other strains. These areas are likely to prove fruitful areas of investigation for future research and will give insights into host pathogen interactions. The findings outlined here and the results of future research will allow design of appropriate intervention strategies for control of this important zoonotic disease.

Chapter 7

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